THE UNIVERSITY
of EDINBURGH

Transcriptomic analysis of sheep macrophages and their response to lipopolysaccharide

Mary Elizabeth Blanche McCulloch

Thesis submitted for the degree of Doctor of Philosophy,

College of Medicine and Veterinary Medicine

University of Edinburgh

July 2018

Declaration

I declare that this thesis presented for the degree of Doctor of Philosophy at the College of Medicine and Veterinary Medicine, University of Edinburgh, has been composed by myself and help from other people is clearly acknowledged. I confirm that the work has not been submitted for any other academic degree or professional qualification at this University or any other institution.

Mary McCulloch

July 2018

Abstract

Sheep are ruminant animals, highly adapted to exploit pastures of low biological value and an economically important livestock species. They also provide a useful animal model for multiple areas of human medicine. Their productivity is compromised by many viral, bacterial, fungal, protozoan and helminthic pathogens. The innate immune system evolves under stringent selective pressure from pathogens. In this project, I aimed to identify genes involved in innate immunity in sheep by dissecting the transcriptome of sheep macrophages. The analysis focussed on identification of genes that may be associated with either disease susceptibility or resistance traits. The project formed part of the broader transcriptomic atlas for sheep.

A method was established and validated for the production of sheep bone marrow derived macrophages (BMDMs) grown in macrophage colony-stimulating factor (CSF1). These cells responded to bacterial Lipopolysaccharide (LPS), an archetypal agonist of the pattern recognition receptor, TLR4, with induction of inflammatory cytokines, but unlike rodent macrophages, sheep produced no nitric oxide. Bone marrow-derived macrophages were produced from male (n=3) and female (n=3) Scottish Blackface X Texel animals used in The Sheep Atlas project, and mRNA was isolated from the cells at 0, 2, 4, 7 and 24 hours following stimulation. Two different protocols of mRNA preparation were used. For 0 and 7 hour samples, samples were depleted of rRNA, and RNA-Seq was carried out at a depth of 100 million reads. For all samples in the time course (including 0 and 7), mRNA was prepared by polyA selection, and RNA-Seq was performed at lower depth (25 million reads) and a detailed analysis of the different outcomes is presented.

Two pipelines, Kallisto and StringTie were used to identify and quantify transcripts in the LPS time course transcriptomic data, along with other subsets of innate immune cells from the wider atlas. The former pipeline provides quantitative known transcript expression estimates, the latter generates novel transcript and gene models. Analysing the transcriptional signatures of these samples provided insight into the metabolic, regulatory and innate response pathways that sheep share with other animals, with both Myeloid differentiation primary response 88 protein (MYD88) dependent and independent pathways being activated following LPS stimulation, with hundreds of the same downstream cytokines being highly expressed in response, such as TNF and many interleukins as seen in other species innate responses. It has also highlighted aspects of the

response that separate sheep from other animals, such as their metabolism and biosynthesis of steroid and tryptophan as well as demonstrating differences in specific expression of certain genes. Two highly regulated and expressed genes noted during the peak response at 7hrs (ENSOARG00000005159 and ENSOARG00000006889), were both assigned a functional annotation as being the protease inhibitor Serpin family B member 2 gene (*SERPINB2*). This appears to be a gene duplication. This sheep expression profile of *SERPINB2* is shared with mice but distinct from humans and pigs. Similar to other animals, individual animals were found to vary markedly in their transcriptional response to LPS, demonstrating hyper, early and delayed responses between the individuals.

The current reference transcriptome OarV3.1 contains 28757 transcripts, of which only 18488 are functionally annotated. More than 85% of all reference transcripts were detected in sheep macrophages and over 300 candidate annotations for genes identified only by Ensembl sheep (Ovis aries) gene ID (ENSOARG) numbers were manually assigned a functional annotation by clustering co-expression estimates using the network analysis tool, Miru and inferring function by 'guilt by association' of these unknown genes which demonstrated similar expression profiles to those with known function. The nearest orthologues and synteny with other species were used to validate these suspected annotations. A select few examples include PYD and CARD domain containing gene (*PYCARD*), all the early growth response factors and many of the C-X-C Motif chemokines.

In summary, this project identified multiple sheep-specific aspects of the innate immune response, whilst assigning hundreds of genes a functional annotation and detecting new transcript models for multiple known genes. Many regulated, highly expressed, multi exoned, novel gene models have also been identified which are worthy of future investigation.

Lay Summary

Macrophages are cells of the immune system that are found in every tissue in the body. Their main function is to recognise and destroy pathogens that cause infectious disease. Every species has evolved to deal with specific infectious challenges and as a consequence, the genes that are needed for effective immunity vary between species and between individuals. Variation between individuals explains differences in disease resistance.

Sheep are ruminant animals and an important livestock species. This project aimed to identify the sets of genes that are required for effective immune defence in the sheep, and to dissect the ways in which sheep differ from other animals. The outcomes will support effective breeding of sheep with greater resistance to infectious disease.

Acknowledgements

Professor David Hume, **Professor Kim Summers** and **Dr James Prendergast**. I would never have got through this without you all and this thesis owes everything to your brilliance. I will always be in awe of your knowledge and the patience you have all shown me in your teaching and the help given in editing this thesis. Thank you for being so generous with the time you gave, the remarkable kindness and the support that you have shown me throughout my time undertaking this PhD, which without doubt has been the toughest challenge of my life.

Professor David Hume, it is hard to articulate the lasting impression and impact you have had on me. Your phenomenal brain and scientific knowledge have no limits, yet you have always been so understanding, patient and encouraging of the crumbling, sleep deprived and hormonally challenged brain that has been mine for much of this PhD. Thank you so much for all your teaching, for sharing your love for macrophages and innate immunity, your excitement and enthusiasm for all genes and species, and for all the editing and amazing, generous help you have given in producing and bringing together this thesis. I am incredibly grateful to you for allowing me to join team Sheep.

Professor Kim Summers, thank you for all the help, support, kindness and understanding you have given me in supervision of my project and with the production of this thesis, as well as on a personal level. Your clarity of explanation, teaching and insight as to where and when help is needed, your open door and your generosity with time is all greatly appreciated. Thank you for sharing your incredible eye for detail and way with words, and for all the encouragement you have given.

Dr James Prendergast, thank you for introducing me to the world of computers, your patience, kindness and understanding with teaching me from scratch and for sharing a fraction of your amazing bioinformatic skills and knowledge. I am so grateful for all your explanations and help when things went wrong. Your remarkable ability to solve problems and find solutions to 'bugs' when they arose will be forever remembered and much appreciated.

Thank you to Professor David Argyle of R(D)SVS for being instrumental in my decision to undertake this PhD, your initial encouragement and for organising the funding, which in partnership with Professor David Hume and The Roslin Institute made this PhD possible.

Thank you to my thesis committee for their encouragement and support which includes Dr Gerry McLachlan and Dr Fiona Houston.

Dr Emily Clark, I am forever grateful for all your help, wise words and calmness throughout my time at Roslin, and your incredible organisational skills, gentle understanding and your kindness and generosity with offering help and teaching, all of which have made a lasting impression. I will always be incredibly grateful for everything you did to help and support me in the lab upon my return. Dr Steve Bush, thank you for sharing your enthusiasm for bioinformatics, the many tricks and some of the magic that you know (highlighting, the right click, the double click and the need for organisation, notepad and screens all made a massive difference – thank you!). Dr Clare Pridans, who knows everything about keeping macrophages happy in culture, Dr Kristin Sauter and Dr Rachel Young, thank you all for introducing me (as a rough and not so ready, aged vet from practice) to scientific laboratory work, code, conduct and equipment. Thanks to your teaching, knowledge and expertise, the data for this project was created and everything and one is still in one piece – thank you.

Thank you also to the many members of the Hume lab and Roslin institute who I would like to mention as they have helped, supported and encouraged me in this project and my time at Roslin; Iseabail Farquhar, Lindsey Waddell, Lucas Lefevre, Dr Anna Raper, Dr Tim Regan, Dr Rocio Rojo, Dr Sara Clohisey, Charity Muriuki, Dr Zofia Lisowski, Dr Mark Barnett, Ailsa Carlisle, Dr Kay Boulton, Dr Carys Pugh and Dr Stefan Hoj-Edwards. Thank you to Tim King, Peter Tennant and Adrian Ritchie from Dryden farm for your initial help on farm.

A special thanks to Dr Fiona Allan and Dr Iveta Gazova for your understanding, kindness and encouragement and all the time given to me and my family with writing up this thesis. Thank you Norman Russell for generating the beautiful photos of the Atlas Sheep, including my favourite which I hope makes the perfect finish to this thesis.

There are many people that I owe a huge debt of gratitude to for their continued personal support, friendship and encouragement: Professor Simone Meddle, thank you for all your listening, advice and the many dog walks which have literally been my salvation. You are such an inspiration, always kind, understanding and with boundless enthusiasm. Dr Susan Kempson, thank you for your encouragement and support of my return from vet practice to academia and Dr Cali Ingham for your continued support and honesty with how tough balancing life would be/ has been and all the kindness and support you have given me. Dr

Thalia Blacking, Dr Guraa Bergkvist, Dr Erika Abbondati and Dr Jorge Del-Pozo you have all been so supportive, kind and most brilliant friends throughout, thank you all so much.

So many local friends and neighbours have shown remarkable kindness, support and helped when possible; Emma and Robin Hutt, you are the greatest neighbours anyone could wish for. Cathi Gibson, Belle Dieckfoss and Claire Witney, thank you for helping look after Kitty when you did and for your kindness, support and encouragement for me to 'keep going' and just 'get it finished'.

My long suffering, wonderful family; without your loyalty, support, love, encouragement and kindness this PhD would never have been completed. My mother Susan Davidson, father Roderick Davidson and dear brother Euan Davidson, and my parents-in-law, John and Cicely McCulloch, you all know just how much you have helped us as a family get through this. Thank you for always being there when we needed you and for all the help, support and kindness you have given. Last but by no means least: my beloved, loving, patient, supportive and kind, dear husband Robert, and my greatest achievements and most treasured and loved little people, Roddy and Kitty. This thesis is dedicated to all of you.

Publications

Pridans C., Davis G.M., Sauter K.A., Lisowski Z.M., Corripio-Miyar Y., Raper A., Lefevre L., Young R., **McCulloch M.E.**, Lillico S., Milne E., Whitelaw B. & Hume D.A. (2016). A *Csf1r*-EGFP Transgene Provides a Novel Marker for Monocyte Subsets in Sheep. *J. Immunol.* 197(6): 2297–2305.

Sauter K.A., Waddell L.A., Lisowski Z.M., Young R., Lefevre L., Davis G.M., Clohisey S.M., **McCulloch M.E**., Magowan E., Mabbott N.A., Summers K.M. & Hume D.A. (2016) Macrophage colony-stimulating factor (CSF1) controls monocyte production and maturation and the steady-state size of the liver in pigs. *Am. J. Physiol. Gastrointest. Liv. Physiol.* 311(3):G533-547.

Bush S.J., **McCulloch M.E.**, Summers K.M., Hume D.A. & Clark E.L. (2017) Integration of quantified expression estimates from polyA-selected and rRNA -depleted RNA-seq libraries. *BMC Bioinform.* 18(1):301.

Banos G., Bramis G., Bush S.J., Clark E.L., **McCulloch M.E.**, Smith J., Schulze G., Arsenos G., Hume D.A. & Psifidi A. (2017) The genomic architecture of mastitis resistance in dairy sheep. *BMC Genomics.* 18(1):624.

Clark E.L., Bush S.J., **McCulloch M.E.**, Farquhar I.L., Young R., Lefevre L., Pridans C., Tsang H.G., Wu C., Afrasiabi C., Watson M., Whitelaw C.B., Freeman T., Summers K.M., Archibald A.L. & Hume D.A. (2017) A high resolution atlas of gene expression of the domestic sheep (Ovis aries). *PLoS Genetics.* 13(9):e1006997

Bush S.J., Muriuki C., **McCulloch M.E.**, Farquhar I.L., Clark E.L., Hume D.A. (2018) Cross-species inference of long non-coding RNAs greatly expands the ruminant transcriptome. *Genetics Selection Evolution.* 50:20.

Young R., Bush S.J., Lefevre L., **McCulloch M.E.,** Lisowski Z.M., Muriuki C., Waddell L.A., Sauter K.A., Pridans C., Clark E.L & Hume D.A. (2018) Species-specific transcriptional regulation of genes involved in nitric oxide production and arginine metabolism in macrophages. *ImmunoHorizons.* 2(1):27-37

Waddell L., Lefevre L., Bush S.J., Raper A., Young R., Lisowski Z.M., **McCulloch M.E.**, Muriuki C., Sauter K.A., Clark E.L., Irvine K.M., Pridans C., Hope J & Hume D.A. ADGRE1 (EMR1, F4/80) is a rapidly-evolving gene expressed in monocyte-macrophages in all mammalian species. Submitted to *Frontiers in Immunology* June 2018

Bush S.J., **McCulloch M.E.**, Muriuki C., Salavati M., Davis G.M., Farquhar I.L., Lisowski Z.M., Archibald A.L., Hume D.A. & Clark E.L. Comprehensive transcriptional profiling of the gastrointestinal tract of ruminants from birth to adulthood reveals strong developmental stage specific gene expression. Submitted to *G3* July 2018

List of Figures

List of Tables

List of Abbreviations

AM	alveolar macrophage
bp	base pair
BL	blood leucocyte
CD	cluster of differentiation
CSFs	colony stimulating factors
CLRs	C-type lectin receptors
OarV3.1	current sheep genome assembly
DAMPs	damage-associated molecular patterns
DC	dendritic cell
DNA	deoxyribonucleic acid
ERV	endogenous retrovirus
ER	endoplasmic reticulum
FDR	false discovery rate
FACS	fluorescence-activated cell sorting
FPKM	fragments per kilobase of exon per million reads mapped
FFAR	free fatty acids receptor
FANTOM	Fuctional annotation of mammalian genome
FAANG	Functional Annotation of Animal Genomes
GO	gene ontology
GTF	gene transfer format
GC	glucocorticoid
IRFs	interferon regulatory factors
ISGS	International Sheep Genomics Consortium
KO	knockout
LRRs	leucine-rich repeats
LPS	lipopolysaccharide
lincRNA	long intergenic non-coding RNA
lncRNA	long non-coding RNA
MHC	major histocompatibility complex
mRNA	messenger RNA

miRNA	micro RNA
MtRNA	mitochondrial RNA
MDM	monocyte derived macrophages
MPS	mononuclear phagocyte system
NLRs	nod-like receptors
PAMPs	pathogen-associated molecular patterns
PRRs	pattern recognition receptors
PBMCs	peripheral blood mononuclear cells
polyA	polyadenylation (long runs of adenosine residues)
PCA	principal component analysis
ROS	reactive oxygen species
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RLRs	RIG-I like receptors
RINe	RNA Integrity number equivalent
RNA-Seq	RNA sequencing
SINEs	Short interspersed nuclear elements
SNPs	single nucleotide polymorphisms
SNVs	single nucleotide variants
snRNAs	small nuclear RNAs
snoRNAs	small nucleolar RNAs
TLRs	toll like receptors
TF	transcription factor
TPM	transcripts per million
tRNAs	transfer RNAs
TCA	tricarboxylic acid
VFAs	volatile fatty acids

Contents

Chapter 1 Introduction

The immune response involves the temporal induction and repression of thousands of genes, and has evolved to protect all living organisms from both internal and external threats to their survival, under intense selective pressures from pathogens (Kimbrell & Beutler, 2001), whilst tolerating the independent co-evolution of beneficial host-microbe relationships (Bishop, 2015). Sheep are man's most ancient provider, being the first domesticated livestock species more than 12, 000 years ago (Larson *et al.*, 2014), and as ruminant animals their immune response has evolved alongside a symbiotic relationship with their fermentative rumen microbiome, which is thought to have evolved 35-40 million years ago (Hackmann & Spain, 2010). The products of fermentation are vital in meeting the sheep's metabolic needs (Shabat *et al.*, 2016).

Mice have dominated immunological research to date, being the most commonly used animal model for human medicine (Perlman, 2016), however there are significant differences between mice and humans in how these species respond to the same immunological stimuli (Schroder *et al.*, 2012). There is divergence in the response between different mouse strains and between individual mice (Raza *et al.*, 2014) and human individuals vary in their immune response (Brodin & Davis, 2017). Different breeds of pig and individual pigs can vary in their response but pigs have been found to resemble humans more closely than mice (Kapetanovic *et al.*, 2012, Kapetanovic *et al.*, 2013). As sheep depend upon their microbiome to a greater extent than any of these monogastric mammals, it is expected that their immune response will vary between individuals and be different to that of monogastric animals.

1.1 Innate immunity: function and overview

The mammalian immune response is dependent upon regulation of a careful interplay between two broad, complementary arms of defence, innate and adaptive immunity (Paul, 2011). The innate immune system is evolutionarily ancient, rapid, independent of antigen and provides the first line of defence for all single cell organisms and multicellular classes of plants and animals (Tauber, 2003, Beutler, 2004). The adaptive immune system evolved more recently, being restricted to vertebrates with jaws (gnathostomes) (Cooper & Alder,

2006), and is highly specific, adaptable and demonstrates classical immunological memory (Ehrlich, 1900, Litman *et al.*, 2010).

Adaptive immunity is mediated by B and T lymphocytes of the lymphoid system (Alberts *et al.*, 2002). Innate immunity is mediated by cells of the mononuclear phagocyte system (MPS), which encompasses bone marrow pluripotent cells, blood monocytes, dendritic cells (DCs) and tissue macrophages. Cells of the MPS share similar morphology (stellate with endocytic activity), are capable of specific enzyme production for example non-specific esterase, lysosomal hydrolases and ectoenzymes, and have the ability to phagocytose 'foreign' particles such as latex, colloidal carbon and display endocytic receptors for Fc and complement (Hume *et al.*, 2002, Hume, 2006). Cells of the innate immune system interact with the lymphoid system at many levels, and the lymph nodes themselves provide clearance of dead macrophages, which has been extensively reviewed (Zhang *et al.*, 2016, Gordon & Pluddemann, 2017).

Function 1 of the immune system is to distinguish between self and non-self, whilst recognising self-cells that have become abnormal and therefore present a threat to survival. For example cancerous cells, damaged cells or cells containing intracellular pathogens, have to be distinguished from normal self-cells, and non-self-cells which are desirable or vital for the host such as gut microbiota, have to be distinguished from pathogens (Brestoff & Artis, 2013). This function is mediated through the process of tolerance which establishes the key hallmarks of self-cells during development and by interaction between the innate and adaptive immune systems (Mowat, 2003). Professional phagocytic cells play a major role, as they are able to recognise, capture, process, and present potential antigens to cells of the adaptive immune system, driving the activation, expansion and differentiation of B and T lymphocytes which improve clearance of the pathogen (Batista & Harwood, 2009). Thus, the innate immune system not only functions as the first line of defence, initiating the inflammatory response, but also provides protection whilst adaptive responses develop and influences the nature of adaptive responses (Janeway, 1989, Palucka & Banchereau, 1999, Pasare & Medzhitov, 2005, Paul, 2011). Indeed, vaccine design is based upon a detailed understanding of this innate immune signalling (Sundling & Sandgren, 2016).

Function 2 of the immune system is to maintain immunological memory so that when a pathogen is encountered again the system can respond rapidly and efficiently. Whilst this is

primarily mediated through the adaptive immune system, the innate immune system has also been found to develop "trained immunity", through transcriptional and epigenetic reprogramming (although shorter lived and less specific than that of the adaptive immune system), when a pathogen is reencountered (Netea *et al.*, 2011, Netea *et al.*, 2016). Epigenomics encompasses the complexities of chromatin architecture and the precise physiological targets of regulatory proteins involved in recognising, depositing and removing chemical modifications from chromatin, as well as other factors which can alter gene expression (van der Heijden *et al.*, 2018). Initial evidence for this arose from studies which found murine macrophages developed tolerance to the bacterial endotoxin, lipopolysaccharide (LPS) at the molecular level, where gene-specific chromatin modifications were responsible for the transient silencing of certain groups of genes whilst priming other groups (Foster *et al.*, 2007). More recently the mouse microbiome has been found to be responsible for long-term functional reprogramming of bone-marrow derived DCs, which overall shows that communication from the microbiome to the bone marrow can have significant immunomodulatory effects (Burgess *et al.*, 2014). Trained innate memory has also been suggested as a contributing factor to chronic immune-mediated chronic diseases such as atherosclerosis (van der Heijden *et al.*, 2018).

The organised system of the innate response is evolutionarily ancient and vital in ensuring the survival of all multicellular organisms, hence the system is highly optimized by natural selection and the Red Queen co-evolution between hosts and parasites. Two Red Queen processes are known to provide the extremes of the spectrum of co-evolution; the Red Queen arms race which is driven by positive selection and reduces genetic variation, and Red Queen dynamics which preserves genetic variation for the long-term (Lighten *et al.*, 2017). Genes available to a given species have often been lost or changed function through evolution (Viljakainen, 2015). The molecular mechanisms for this evolution may involve base substitutions, gene duplications, deletions, alternative splicing, gene recombination, domain shuffling, retrotransposition, and gene conversion (Buchmann, 2014). The patterns of these changes increase understanding of the evolutionary dynamics between host and pathogen. This thesis presents a study of sheep innate immune genes to add to this understanding.

Herbivorous ruminant animals such as sheep are particularly dependent upon their diverse gut microbiota (bacteria, fungi and protozoa) to convert complex carbohydrates such as

cellulose into volatile fatty acids (VFAs) for ruminant metabolism (Doreau & Ferlay, 1994). Cellulose represents the most abundant biopolymer on earth, a highly specific and architecturally diverse macromolecule made of up of repeating glucose units (Klemm *et al.*, 2005). The sheep immune system must be able to distinguish subtle differences between desirable and undesirable components, so that only organisms and compounds that pose a true risk to the animal are attacked rapidly and efficiently. Therefore, the ruminant immune system must be highly specialised, and important evolutionary strategies could be discovered by comparing different ruminants with each other and non-ruminant animals.

1.2 Major macrophage functions

Macrophages are professional phagocytes that were first recognised by Elie Metchnikoff (the father of innate immunity) in 1882, who revealed how important phagocytosis is in development, homeostasis and disease (Gordon, 2016). They represent the tissue compartment of the MPS. They are situated throughout the body, in every mammalian tissue, and strategically are most concentrated at areas of greatest risk of invasion of pathogens, lining all epithelia and microvasculature, which ensures they are the first line of defence (Hume *et al.*, 2002, Hume, 2006).

Their exquisite sensitivity to their microenvironment is achieved through a diverse repertoire of surface receptors which are expressed in infinite combinations, with interaction between ligand and receptor leading to a cascade of gene transcription, changing the gene expression pattern to result in “activation”(Mosser & Edwards, 2008, Jenkins & Hume, 2014, Gordon & Pluddemann, 2017). Any gene expression program can be fixed in certain tissue macrophages and induced in others, depending on what is required and the surrounding microenvironment, which means that the resident tissue macrophage becomes increasingly adapted in a dynamic and continuous way to its microenvironment (Wynn *et al.*, 2013, Hume, 2015).

As well as surveillance and phagocytosis of microbes and dead/damaged/dying cells, activation of macrophages involves the acquisition of microbicidal effector functions, the secretion of proinflammatory cytokines and the downstream recruitment of other immune cells (Adams, 1989, Schroder *et al.*, 2004). Macrophages process and recycle compounds such haemoglobin and iron (Knutson & Wessling-Resnick, 2003). Cytokines released by macrophages in response to stimuli have local and systemic effects, influencing cellular proliferation, differentiation, acquired immunity, embryonic development and wound

repair, which means that as a cell type, macrophages are akin to a disperse homeostatic organ (Gordon & Pluddemann, 2017) which largely dictates the outcome of any disease (Murray & Wynn, 2011, Gordon *et al.*, 2014, Hume, 2015, Gordon & Pluddemann, 2017).

Their broad function, anatomical diversity and plasticity is owed to an equally diverse, complex and highly regulated transcriptome. The cascade of activation and downstream transcription involves thousands of genes being tightly regulated in organised sequential waves of expression, either being induced and increasing in expression or being repressed and decreasing, involving multiple signalling pathways and overall creating a complex regulatory expression network. Feedback control genes are induced in conjunction with proinflammatory genes, acting at every possible level of the cascade (Hume, 2008, Biswas & Mantovani, 2010, Gautier *et al.*, 2012, Wynn *et al.*, 2013, Gordon *et al.*, 2014, Jenkins & Hume, 2014, 2015, Hume, 2015).

Activation of macrophages and the downstream inflammatory response does result in a transient decline in tissue function. This results in some undesirable aspects of the stress response, including heat shock, hypoxia, high levels of reactive oxygen species and glucose and amino acid deprivation. These aspects affect cellular function and the capacity to switch metabolic homeostatic set points (Medzhitov, 2010). By comparing species and individuals in their macrophage transcriptional response to stimuli, causative genetic variations between species, breeds and individuals may help explain differences between species and individuals in susceptibility and resilience to disease.

1.2.1 Pattern recognition receptors and surface receptors of macrophages

Classic macrophage cell surface receptors are shown in Figure 1 (taken from (Gordon & Pluddemann, 2017)) and include F4/80(ADGRE1), CD68, CSF1R, MERTK and CD64 (which can all also serve as surface markers for immunocytochemistry or fluorescence-activated cell sorting (FACS) analysis). Numerous receptors/markers mediate phagocytosis of a wide range of particles including apoptotic cells (such as CD36, SRA and TIM4), circulating ligands (such as CCR2 which detects the chemokine MCP-1), growth factors (colony stimulating factors (CSFs)), sialic acid-binding immunoglobulin-like lectin 1 (SIGLEC1 / CD169), which along with CD36, play important roles in adhesion, and scavenger receptors such as SR-A which internalises polyanionic ligands (Gordon & Pluddemann, 2017). The large repertoire of receptors summarised in Figure 1.1 (taken from (Gordon & Pluddemann, 2017)) reflects the multiple complex functions of macrophages.

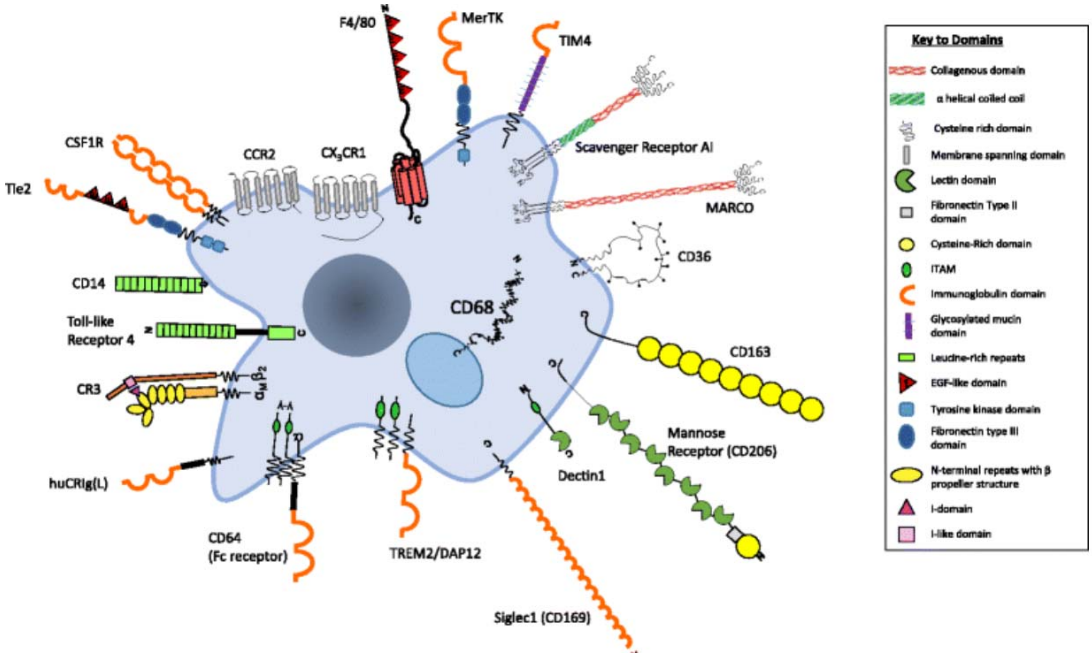


Figure 1.1 Summary of some of the diverse plasma membrane receptors of macrophages (taken from (Gordon & Pluddemann, 2017)).
Receptors involve a range of structural domains, described in the key.

Surveillance and recognition of particles is achieved through the expression of conserved germline encoded pattern recognition receptors (PRRs), first identified over 30 years ago (Janeway, 1989). PRRs recognise endogenous and exogenous conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), flagellin and lipoproteins, and damage-associated molecular patterns (DAMPs), associated with host danger signals, which are released in response to stress, tissue damage and necrotic cell death (Akira *et al.*, 2001, Gordon, 2002, Janeway & Medzhitov, 2002, Ravasi *et al.*, 2002, Hoffmann, 2003, Taylor *et al.*, 2005, Akira *et al.*, 2006, Hume, 2008). Following phagocytosis or endocytosis, pathogens may enter cells and become a target for a wide array of intracellular recognition receptors (Schroder & Tschopp, 2010, Patel & García-Sastre, 2014).

PRRs include toll like receptors (TLRs), RIG-I like receptors (RLRs), nod-like receptors (NLRs), C-type lectin receptors (CLRs) and the intracellular DNA sensors DAI and AIM2 which have been extensively reviewed (Akira *et al.*, 2006, Schroder & Tschopp, 2010). CLRs such as macrophage mannose receptor (MMR/MRC1) and attractin (CD93/C1QR1), which are expressed on the cell surface as transmembrane proteins, can be secreted as molecules,

which are able to recognise carbohydrates, proteins, lipids and even inorganic components such as ice (Brown *et al.*, 2018).

PRRs can act alone or in concert, with the sequential detection of pathogens by different receptors in different cellular compartments resulting in a complex interplay of diverging downstream signalling cascades, that tailor the response specifically to the particular class of microbe (Franchi *et al.*, 2008, Shaw *et al.*, 2008). Many of these signalling pathways are highly conserved, such as the activation of transcription factors nuclear factor-[kappa]B (NFKB) and Activator protein 1 (AP-1) which lead to inflammatory cytokine expression. Interferon regulatory factors (IRFs) lead to type I interferon production and prime and activate adaptive responses. Together these instruct the host response to infection or injury (Janeway & Medzhitov, 2002, Akira *et al.*, 2006, Kawai & Akira, 2010, Takeuchi & Akira, 2010).

1.2.2 Toll like receptor (TLR) signalling

Vertebrate toll like receptors (TLRs) are evolutionarily conserved both in coding sequence and signalling pathways and function (Kim & Ausubel, 2005, Roach *et al.*, 2005). They are type I transmembrane receptors, present on the cell surface and intracellularly on the endoplasmic reticulum (ER), endosome, lysosome or endolysosome, and their importance, signalling and function in both innate immunity and in regulating activation of adaptive immunity has been extensively reviewed (Akira *et al.*, 2001, Schnare *et al.*, 2001, Gordon, 2002, Akira *et al.*, 2006, Chang, 2010, Kawai & Akira, 2011, Broz & Monack, 2013, Takeda & Akira, 2015). TLRs recognise a wide range of lipids, lipoproteins, proteins and nucleic acid ligands. They were first identified with their involvement in dorsoventral patterning in the development of *Drosophila* flies, but it was later discovered that they were important in the response to fungal infection of *Drosophila* (Lemaitre *et al.*, 1996) and then the human homologue of the *Drosophila* toll protein was shown to induce the expression of inflammatory cytokines via the NF-KappaB (NFKB) pathway as well as the expression of the co-stimulatory molecule B7.1, required for activating the adaptive immune response (Medzhitov & Janeway, 1997).

TLRs are known to be involved in many vertebrate diseases (Arbour *et al.*, 2000, Nischalke *et al.*, 2011, Piñero *et al.*, 2017). They are central to intestinal development and protection,

as commensals are recognised under homeostatic conditions and TLRs regulate the critical intestinal epithelial barrier (Rakoff-Nahoum *et al.*, 2004). TLR polymorphisms directly affect signalling, with reduced signalling predisposing to many diseases and health concerns including septic shock and gram negative bacterial infections (Lorenz *et al.*, 2002), inflammatory bowel disease and ulcerative colitis (Torok *et al.*, 2004), meningococcal infections (Smirnova *et al.*, 2003), premature birth (Lorenz *et al.*, 2002), leprosy and mycobacterial infections (Bochud *et al.*, 2003) to name but a few, yet a decreased risk of atherogenesis, possibly due to a diminished inflammatory response and so lower concentrations of inflammatory cytokines (Kiechl *et al.*, 2002, Miller *et al.*, 2005).

TLRs all consist of an ectodomain containing varying numbers of leucine-rich repeats (LRRs), which is responsible for recognition of the PAMP/DAMP. The transmembrane domain has a role in receptor oligomerization, important for assembly and function of TLRs (Godfroy *et al.*, 2012). The conserved cytoplasmic tail with a Toll/interleukin-1(IL1) receptor (TIR) domain in the cytoplasm recruits required signalling molecules (Akira *et al.*, 2006), which in turn activate transcription factors including NFKB, IRFs, and MAP kinases which dictate the downstream inflammatory response and signalling, involving cytokines, chemokines and type I IFNs (Akira & Takeda, 2004, Kawasaki & Kawai, 2014).

Adaptor proteins define the particular biological response of any TLR (Yamamoto *et al.*, 2004). There are five important adaptor proteins involved in TLR signalling which have been extensively reviewed (O'Neill & Bowie, 2007, Belinda *et al.*, 2008). All are recruited for signalling via the Toll/interleukin-1 (IL-1) receptor (TIR) domain (Xu *et al.*, 2000).

1) Myeloid differentiation primary-response protein 88 (MyD88), which is utilised by all TLRs except TLR3, and is also found to be involved in IFN gamma, IL1 and IL18 signalling (Lord *et al.*, 1990, Hultmark, 1994, Medzhitov *et al.*, 1998).

2) TIR-domain containing adaptor protein (TIRAP/MAL) is a sorting adaptor that recruits MyD88. It is required by TLRs 1, 2, 4 and 6, but also endosomal TLR9, which demonstrates how this adaptor can bind to different lipids and so initiate specific transduction pathways (Kagan & Medzhitov, 2006). It can also be degraded by suppressor of cytokine signalling 1 (SOCS1) (Horng *et al.*, 2002, Yamamoto *et al.*, 2002).

3) TIR-domain containing adaptor protein inducing interferon β (TRIF), which is utilised by TLRs 3 and 4 and which activates IRF3 and NFKB and mediates apoptosis (Yamamoto *et al.*, 2002, Yamamoto *et al.*, 2003).

4) TRIF-related adaptor molecule (TRAM), which is required by TLR4 to recruit TRIF and regulated by protein kinase C (Fitzgerald *et al.*, 2003, Yamamoto *et al.*, 2003).

5) Sterile α- and armadillo-motif-containing protein (SARM), which has been shown to negatively regulate TRIF and so signalling of TLR3 and TLR4 (Mink *et al.*, 2001, Couillault *et al.*, 2004, Carty *et al.*, 2006).

There are 6 major families of vertebrate TLRs, 10 TLR genes that have been recognised in humans and sheep and 12 TLR genes in mice, 9 receptors encoded in the *Drosophila* genome, and 20 putative TLR variants in zebrafish (Roach *et al.*, 2005, Li *et al.*, 2015). This repertoire is very distinct from invertebrates, with 253 TLR genes that have been detected in the purple sea urchin (*Strongylocentrotus purpuratus*) genome (Buckley & Rast, 2012, Kawasaki & Kawai, 2014). Strong selective pressures have resulted in these different families of TLR, which vary in length in their LRR domains and the general class of PAMP recognised. Vertebrate TLRs produce a "star phylogeny", implying that they are all evolving at about the same slow rate. Selection for maintaining a functional endogenous signalling network (such as conserving binding with accessory molecules like CD36 or cytokines such as IL1B) appears to be the strongest evolutionary pressure, which is unusual for multigene families, where often some members derive new functions (Roach *et al.*, 2005).

TLRs 3, 7, 8 and 9 are all expressed on endosomal membranes inside cells, so their ligands require internalisation. Other TLRs are expressed on the cell surface (TLRs 1, 2, 4, 5, 6, 10, 11, 12). This distribution throughout the cell and the trafficking from the ER to the Golgi to the specific membrane for expression, is critical for PAMP recognition and determining self from non-self which otherwise leads to autoimmunity (Tabeta *et al.*, 2006, Lee *et al.*, 2013)

TLRs 1, 2, 4 and 6 recognise bacterial lipids, TLRs 3, 7, and 8 recognise viral RNA, TLR9 recognises bacterial DNA, and TLR5 and 10 recognise bacterial and parasite proteins. TLRs 11 and 12 expressed in mice recognise flagellin and profilin (Koblansky *et al.*, 2013). Enhanced antigen uptake by TLRs is important for presentation (Hemmi & Akira, 2005,

Kawai & Akira, 2010), and each TLR induces distinct patterns of gene expression, involving the same signalling as IL1R (Akira & Takeda, 2004).

Several transmembrane molecules help regulate TLR signalling including a glycophosphatidylinositol-anchored protein CD14, which is a co-receptor with TLR4 and also required for TLR7 and 9 induction of proinflammatory cytokines (Baumann *et al.*, 2010) and MD2 which is needed for LPS recognition and endocytosis (Zanoni *et al.*, 2011).

1.2.3 Intracellular pathogen receptor signalling

Intracellular PRRs that act as cytosolic sensors include retinoid acid-inducible gene-I (RIG-I)-like helicases (RLHs) such as RIG-I and MDA5, DNA sensors such as DAI and AIM2 (Patel & García-Sastre, 2014) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) which act as molecular scaffolds within the cytoplasm and can activate NFKB, MAPKs and control activation of inflammatory caspases which in turn are responsible for the maturation and release of potent cytokines such as IL1B and IL18 (Franchi *et al.*, 2009, Schroder & Tschopp, 2010).

Nod-like receptors (NLRs) represent the largest family of PRRs. There are 23 human genes and at least 34 mouse genes that encode NLRs. These receptors are expressed in the cytosol, primarily in macrophage and neutrophil cells (Franchi *et al.*, 2009). They have multiple N- terminal domains responsible for downstream signalling. These include a caspase recruitment domain (CARD), pyrin domain (PYD), acidic domain, and a NACHT domain which is the only domain shared by all NLRs. The NACHT domain is centrally located, determining 3 families of NLRs; NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1), the NLRPs (NLRP1-14, also called NALPs) and the IPAF subfamily, consisting of IPAF (NLRC4) and NAIP (Schroder & Tschopp, 2010).

The key signalling platform of the innate immune system has been called the inflammasome. Some NLRs are known to provide a scaffold for canonical inflammasome activation, which are then connected to caspase 1 via an adaptor protein ASC (encoded by *PYCARD*). NLRP1, NLRP3 and IPAF are able to activate caspase 1, a proinflammatory caspase which mediates IL1B processing, release and therefore signalling (Martinon *et al.*, 2002) and IL18 (Latz *et al.*, 2013).

RIG-I like receptors (RLRs) are expressed in the cytosol of cells. They are able to sense 'foreign' 5'-triphosphate (5'-ppp) double stranded RNA molecules produced during

infection (as host mRNAs synthesised by RNA polymerase II produce a 5'-ppp end which is then processed to have an added 7-methyl guanosine cap) and following ligation, trigger efficient downstream signalling to mount an antimicrobial response against many viruses and intracellular bacteria (Yoneyama *et al.*, 2004, Patel & García-Sastre, 2014, Bordon, 2015). RIG-1 is a protein belonging to the DExH-box family, and has been shown to have roles in proinflammatory pathways, including the response to bacterial LPS, as it regulates expression of cyclooxygenase-2 (COX-2) (Imaizumi *et al.*, 2002), and the response to IFN-γ, which induces expression of RIG-1 (Cui *et al.*, 2004).

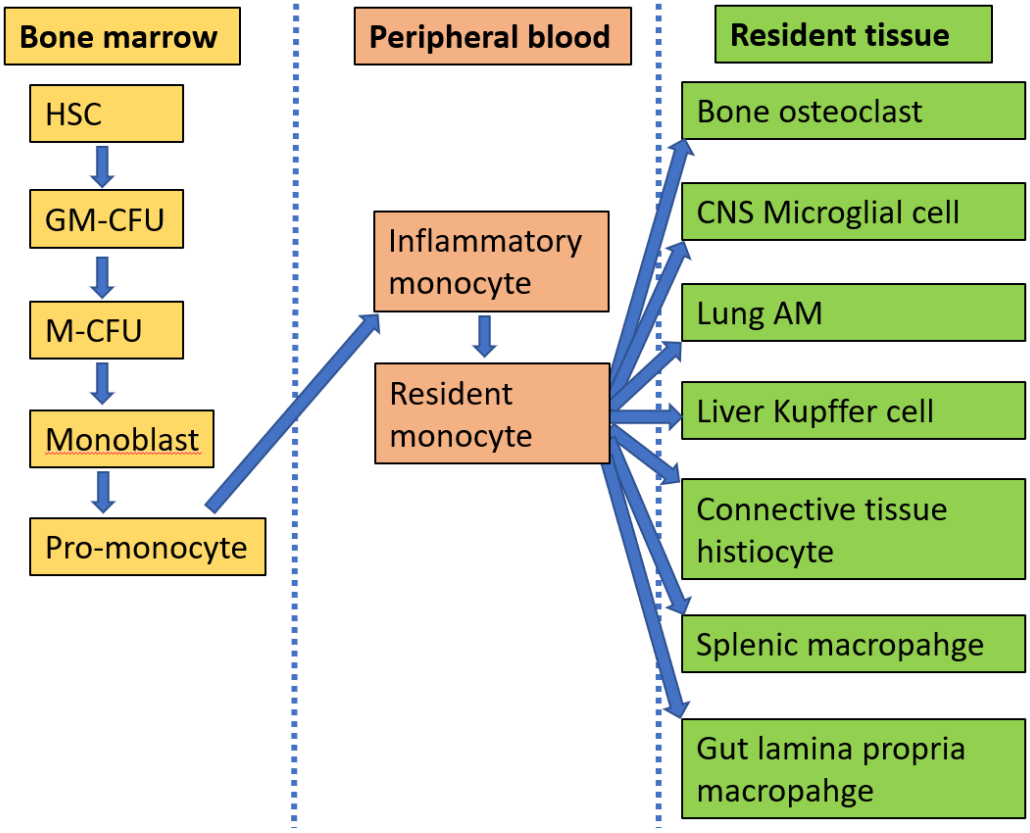
1.3 Macrophage differentiation and colony stimulating factors

1.3.1 Macrophage cell types and origins

During early mammalian embryonic development, the first macrophages appear in the yolk sac (Cuadros *et al.*, 1992, Bertrand *et al.*, 2005). Once circulation is established, haematopoietic stem cells (HSC) from the yolk sac start to colonise the liver, and both yolk sac and liver macrophages seed populations to the brain (microglia), skin (Langerhans cells) and lung (alveolar macrophages), which can self-renew (Kikuchi & Kondo, 2006, Lux *et al.*, 2008, Swirski *et al.*, 2014, Hume *et al.*, 2016). The replenishment of tissue macrophages as to whether they are derived through self-renewal or from circulating monocytes varies between adult tissues, inflammation and malignancy states, promoting the concept of a layered myeloid system (Hashimoto *et al.*, 2013, Bain *et al.*, 2014, Amit *et al.*, 2016, Ginhoux & Guilliams, 2016, Perdiguero & Geissmann, 2016).

In the adult animal, myelopoiesis occurs in the bone marrow, and haematopoietic multipotential progenitor cells commit to the myeloid lineage, differentiating in response to specific haematopoietic growth factors, primarily CSF1 (Sordet *et al.*, 2002) and IL34 (Lin *et al.*, 2008) which has been extensively reviewed (Hume *et al.*, 2002, Hume, 2006, Orkin & Zon, 2008, Wynn *et al.*, 2013) and is summarised in Figure 1.2. Both CSF1 and IL34 share a highly conserved receptor encoded by the c-fms proto-oncogene, Macrophage colony-stimulating factor 1 receptor (*CSF1R*) (Pixley & Stanley, 2004, Chitu & Stanley, 2006, Garceau *et al.*, 2010).

Figure 1.2 Summary of the differentiation of monocytes from bone marrow haematopoietic stem cells (HSC) and their migration and recruitment into different tissues (adapted from (Mosser & Edwards, 2008)).



All monocytes have the ability to differentiate to become macrophages if provided with the correct stimuli (Yona *et al.*, 2013). In humans, so-called classical monocytes possess high levels of the co-receptor for LPS (CD14) and low levels of the Fc receptor CD16. In mice, these cells are defined by high levels of the marker Ly6c. Classical monocytes possess high levels of the CC-chemokine receptor 2 and respond to the ligand CCL2 (monocyte chemotactic peptide 1; MCP1). In response to inflammatory mediators, they are released from the bone marrow and into the circulation for recruitment and differentiation into mature tissue macrophages, which over time then respond to their microenvironment and become ever more specialised and differentiated (Yona *et al.*, 2013, Ginhoux & Guilliams, 2016). Non-classical monocytes (in humans defined by low CD14 and high CD16, and in mice by low Ly6C) express an alternative chemokine receptor, CX3CR1. They are believed to differentiate from the classical cells, are more long -lived, and function mainly as patrolling cells in the blood (Geissmann *et al.*, 2010)

Dendritic cells (DCs) are a family of professional antigen-presenting cells able to prime naïve T cells and many are phagocytic and overlap in function with monocytes and macrophages. DCs are now classified as a separate lineage of the mononuclear phagocyte system (MPS) that have been shown to differ in their growth factor dependence compared to macrophages, depending upon FLT3L (Guilliams *et al.*, 2014, Jenkins & Hume, 2014, Hume, 2015). There are two separate lineages: DCs derived from monocytes and those that arise from a separate adult HSC derived, DC precursor (Guilliams *et al.*, 2014). There are three resident groups of DCs; plasmacytoid DCs (pDCs), migratory DCs and monocyte derived DCs and there are multiple further subsets for each of these groups, differing in the surface markers they express (CD11c, CD8, CD103, F4/80 and CD11b) (Liu *et al.*, 2009, Jenkins & Hume, 2014). Inflammation and the presence of growth factors change the different DC populations and they can be mobilised, activate naïve and memory T cells and secrete inflammatory cytokines; thus they are a very important cell type during inflammatory and autoimmune responses (Wicks & Roberts, 2016). All DCs require Fms-like tyrosine kinase 3 ligand (FLT3L) growth factor for development (Ginhoux *et al.*, 2009), and further differentiation is controlled by transcription factors such as IR8, BAFT3, NFIL3 and IRF4 (Hume *et al.*, 2002, Ginhoux *et al.*, 2009, Guilliams *et al.*, 2014).

1.3.2 Macrophage colony stimulating factors

Haematopoietic growth factors are hormone-like proteins that promote blood cell growth and bone marrow proliferation. Included in this group of proteins are the colony stimulating factors (CSFs), which engage with multipotential hematopoietic stem cell progenitors to induce them to become committed to the myeloid cell line, helping govern myeloid cell survival, differentiation, maturation and proliferation (Ichikawa *et al.*, 1966, Metcalf, 2008, Metcalf, 2016). Each factor also contributes to the host response to injury and infection and may provide future potential therapeutic targets for inflammatory and/or autoimmune diseases and cancer (Hamilton, 2008, Hamilton *et al.*, 2016).

There are several distinct glycoprotein growth factors that can influence the differentiation of macrophages, each with a level of specificity in respect to their target cell;

1) Stem cell factor, (KIT ligand)(Broudy, 1997).
2) CSF1 (macrophage colony-stimulating factor (M-CSF)), the major regulator of macrophage formation as discussed in detail below (Stanley & Heard, 1977).

3) IL34 (Interleukin 34) which shares a receptor with CSF1 (Lin *et al.*, 2008).

4) FLT3L (Fms related tyrosine kinase 3 ligand). Primarily associated with dendritic cells, but the receptor for this ligand is expressed on the earliest myeloid progenitors and a shared macrophage-DC progenitor (Geissmann *et al.*, 2010)

5) CSF2 (granulocyte-macrophage colony-stimulating factor (GM-CSF)), which stimulates bone marrow committed progenitors to become monocytes and macrophages, and functions in the differentiation of antigen presenting dendritic cells (Nimer & Uchida, 1995, Hamilton, 2008), and has many other functions involved in innate and adaptive immunity (Hamilton, 2008, Wicks & Roberts, 2016), for example it is vital in alveolar macrophage processing of surfactant (Metcalf, 2016).

6) CSF3 (granulocyte colony-stimulating factor (G-CSF)), which regulates granulocyte formation, release and survival from the bone marrow (Eyles *et al.*, 2006, Hamilton *et al.*, 2016).

7) IL3 (Interleukin 3: multi-CSF), a powerful CSF *in vitro*, acting in the early stages of differentiation of multiple lineages by promoting survival and proliferation of HSCs and committed progenitors in the bone marrow (Nitsche *et al.*, 2003). IL3 has been found to be associated with immunity to parasites and allergic inflammation(Lantz *et al.*, 1998) and recently it has been found to downregulate matrix metalloproteinases (MMP3 and MMP13), caused by inflammatory cytokines IL1B and TNF in a mouse model of inflammatory arthritis (Kour *et al.*, 2016).

The first act through ligand-dependent tyrosine kinase receptors. Conversely, CSF2, CSF3 and IL3 exert their effects through interacting with a corresponding cell surface receptor, which activates Janus Kinase (JAK)-dependent or Signal Transducer and Activator of Transcription (STAT)- dependent pathways and downstream signalling (Thomas *et al.*, 2015). These factors can work individually or in synergy with one another. For example CSF2 and CSF1 combined can enhance granulocyte-macrophage colony formation (Metcalf,

2016). Other haematopoietic growth factors include stem cell factor (SCF/c-kit/CD117), FLT3 (CD135) and IL34 that also act in a highly specific and efficient manner on their target cells (macrophages and dendritic cells) through the selective expression of corresponding receptors (Maraskovsky *et al.*, 1996, Metcalf, 2008, Jenkins & Hume, 2014). The suppressor of cytokine signalling (SOCS) family of cytokines can modulate and terminate the proliferative responses mediated through the JAK-STAT pathway (Krebs & Hilton, 2001, Metcalf, 2016).

1.3.3 Macrophage colony-stimulating factor (M-CSF/ CSF1)

CSF1 is an essential regulator of macrophage formation, actively committing cells to enter the macrophage lineage and is the universal macrophage obligate growth factor which is found in all tissues and serum. It is a basal regulator of macrophages through a negative feedback loop involving CSF1R mediated endocytosis and intracellular degradation. It is the only pleiotropic cytokine to be expressed at high levels in the blood and ubiquitously expressed by most mesenchymal cells; the other CSFs are only produced in the stimulated state (Bartocci *et al.*, 1987, Hume *et al.*, 2002, Gordon *et al.*, 2014). Mutation of the *CSF1* gene in rats and mice produces a severe loss of macrophages, and many pleiotropic effects on other cell types. CSF1 continues to have a critical function in adults, and when this receptor is blocked, there is rapid depletion of most tissue macrophages (Sauter *et al.*, 2014). There are three forms of CSF1: a cell surface glycoprotein, a secreted glycoprotein and a secreted proteoglycan. The different forms have different effects on macrophages, and this cytokine has very important roles in homeostasis, recruitment and proliferation of macrophages during inflammation (Chitu & Stanley, 2006, Jang *et al.*, 2006, Jenkins & Hume, 2014). Levels of CSF1 can be increased by cells either dying locally, emigrating or mechanisms which block degradation (eg TNF converting enzyme (TACE), produced by activated macrophages cleaves CSF1R, which is the sole uptake/removal mechanism) (Bartocci *et al.*, 1987, Sester *et al.*, 1999, Jenkins & Hume, 2014). Increased CSF1 production acts to replenish tissue macrophages, increasing macrophage tissue densities by increasing the production of macrophages via recruitment of monocytes and CCR2 chemokine receptor ligands (Tagliani *et al.*, 2011) and also by increasing proliferation of tissue resident macrophages. Resident tissue macrophages can also proliferate, above the level induced by CSF1, in response to the cytokine, IL4 (Jenkins *et al.*, 2013) which also directs a specific activation pathway (see below).

1.3.4 Colony Stimulating factor 1 receptor (CSF1R)

The receptor for CSF1 (and IL34), is expressed specifically by macrophage lineage cells (Rojo *et al.*, 2017). Macrophage-specific CSF1R expression involves a conserved regulatory element in the second intron, Fms intronic regulatony element (FIRE), which is crucial in directing macrophage specific transcription (Sauter *et al.*, 2013, Hume *et al.*, 2017, Rojo *et al.*, 2017). CSF1R signalling is required for monocyte and macrophage survival, proliferation and differentiation in every tissue of the body, including microglia, myeloid dendritic cells, osteoclasts, monocytes and all tissue macrophages where it governs their development and function (Sasmono *et al.*, 2003, Wei *et al.*, 2010, Nandi *et al.*, 2012, Hume *et al.*, 2016).

Ligation of CSF1R, with either CSF1 or IL34 (both of which are dimers and interact with two receptor molecules) produces a conformational change, receptor dimerization, autophosphorylation and phosphorylation of downstream molecules which increase gene expression of proteins that will remodel the cytoskeleton, enabling macrophage survival, proliferation, motility and differentiation into the mature macrophage that is able to reach full transcriptional potential including the production of a wide range of cytokines and enzymes (Sweet *et al.*, 2002, Pixley & Stanley, 2004, Stanley & Chitu, 2014). Removal of the CSF1R signal changes gene expression in itself, as macrophages are no longer able to respond to CSF1, so genes such as TLR9 that are normally repressed by CSF1, increase in expression (Sester *et al.*, 2005).

The two ligands, CSF1 and IL34, have no discernible sequence similarity. However they do both show similar helical cytokine folds in their biologically active regions and when IL34 is bound to the N-terminal immunoglobulin domains of CSF1R, it shows some similarity in architecture to that of the CSF1/CSF1R assembly (Ma *et al.*, 2012). CSF1R is the only receptor for CSF1. This is different from IL34 which has been shown to act via CSF1R and via a receptor-type protein- tyrosine phosphatase -ζ (PTP-ζ), a cell surface chondroitin sulphate proteoglycan (Nandi *et al.*, 2013).

1.4 Regulators of macrophage function and their receptors

The plasticity of macrophages means that activation and differentiation are inextricably linked to one another and regulation of macrophage differentiation and transcriptional signalling has been extensively reviewed (Ravasi *et al.*, 2007, Ramsey *et al.*, 2008, Bhatt *et al.*, 2012, Amit *et al.*, 2016, Hume *et al.*, 2016).

Helper T cell-mediated immune responses have been classified into two classes, Th1 and Th2, which are mediated by the production of two critical cytokines, interferon gamma and interleukin 4 (IL4). Each of these cytokines acts on macrophages to produce a polarised gene expression profile, which by analogy has been termed M1 and M2 respectively (Mosser & Zhang, 2008, Wynn *et al.*, 2013, Murray *et al.*, 2014).

Interferons are cytokines with important properties including antiviral (Isaacs & Lindenmann, 1987), antiproliferative and immunomodulatory effects. They signal through transcriptional regulatory pathways such as the JAK-STAT pathway, the mitogen-activated protein kinase p38 cascade pathway and the phosphatidylinositol 3-kinase cascade pathway (Platanias, 2005). Their signalling is integrated with that of multiple other cytokines during the immune response (Schroder *et al.*, 2004).

Interferon gamma (IFN gamma) was first identified in 1965 as an antiviral substance that was produced by stimulated leukocytes (Wheelock, 1965). Originally called macrophage activating factor, it can also be secreted by NK cells, B cells, NKT cells and DCs (Munder *et al.*, 1998). IFN gamma regulates multiple transcription factor genes*, IRF 1, 2* and *9*, along with *STAT1* and *NFKB*, which are involved in co-ordinating the next sequential wave of transcription. In the macrophage the main function of IFN gamma is activation of antimicrobial, antiviral and tumoricidal activity through the induction of reactive oxygen and nitrogen species, as well as increased expression of TLRs and transcription factors for TLR responsive genes, increased antigen processing functions and MHC class I and II molecule presentation (Schroder *et al.*, 2004). IFN gamma is also involved in defence against intracellular bacteria (most notably mycobacteria), fungi and parasites, as well as in anti-viral immunity like both type I and II interferons (Zhang *et al.*, 2008). So called M1, or classically-activated macrophages can be induced by IFN gamma alone, but the response is amplified by co-stimulation with bacterial lipopolysaccharide (discussed below) or other agonists (Martinez *et al.*, 2006). The phenotype includes high expression of inflammatory cytokines such as IL1B, IL6, IL12, IL23 and TNF and other effectors such as CD86, CD64 and CD16, and low expression of anti-inflammatory cytokines such as IL10 (Biswas & Mantovani, 2010).

The biology of M2-like, or alternatively-activated macrophages has been reviewed extensively (Biswas & Mantovani, 2010, Gordon & Martinez, 2010). Broadly-speaking, they represent an anti-inflammatory phenotype observed during allergic inflammation and

helminth infection(Mosser & Edwards, 2008, Mantovani *et al.*, 2013, Van Dyken & Locksley, 2013). In mice, at least, alternative activation generates a shift from nitric oxide production to the metabolism of arginine through arginase (Bronte & Zanovello, 2005). The relevance of this pathway to ruminants, and indeed all large animals, will be discussed further in this thesis.

The simplification of macrophage activation states within the M1 (classically activated macrophages, by IFN gamma or Lipopolysaccaride (LPS))(Schroder *et al.*, 2004) or M2 (alternatively activated macrophages, by IL4) (Lawrence & Natoli, 2011) paradigm is not universally accepted (Hume, 2015). Macrophages respond to many agents other than T cell cytokines to alter their function for specific purposes (Raza *et al.*, 2014) and transcriptomic data has demonstrated that macrophage cells vary widely in function depending upon the individual cell, the dose and combination of agonists and the time following exposure (Hume, 2015).

One of the most clinically important agonists that regulates macrophage function is glucocorticoid (GC). The glucocorticoid receptor (GR) is highly expressed by macrophages, thus they are sensitive to the effects of GC which are anti-inflammatory and immune-suppressive, and oppose the action of CSF1 (Hume *et al.*, 1984). By interacting with glucocorticoid response elements (GRE), GC influence many transcription factors including FOS, JUN, CREB and thus, they fine tune the induction or repression of thousands of genes (Russo-Marie, 1992, Kugelberg, 2014). Mice lacking GR have greater mortality and cytokine release in response to LPS and dexamethasone (a synthetic GC) was identified to act by inhibiting p38 MAPK and therefore downstream targets (Bhattacharyya *et al.*, 2007). The response to GC varies greatly between species, due to gain and loss of GRE in target genes (Jubb *et al.*, 2016)

1.5 Lipopolysaccharide (LPS) recognition and TLR4 activation

Bacteria can be divided into two major groups depending upon the different staining of their cells walls; gram positive and gram negative. The outer membrane of the cell envelope of gram negative bacteria contain the potent endotoxin lipopolysaccharide (LPS), which is the major mediator of pathology in gram negative sepsis. LPS is made of a poly- or oligosaccharide anchored by lipid A, the portion/PAMP recognised by TLR4 which is highly immune stimulatory even at very low concentrations (Miller *et al.*, 2005). Lipid A PAMPs vary in their fatty acid side chains, terminal phosphate residues and associated

modifications, which alter the charge and molecular conformation (Schromm *et al.*, 1998) and have been shown to affect the host response (Schromm *et al.*, 2000).

TLR4 was first identified as the likely human orthologue of the *Drosophila* PRR *Toll* (Medzhitov *et al.*, 1997). The first genetic evidence for TLR4 being the main receptor for LPS was obtained by positional cloning of the gene involved in natural LPS resistance in certain inbred mouse strains (Poltorak *et al.*, 1998, Qureshi *et al.*, 1999). These seminal discoveries led to the Nobel prize in Physiology and Medicine in 2011, being awarded to Bruce A. Beutler and Jules A. Hoffmann for their discoveries concerning the activation of innate immunity and to Ralph M.Steinman for his discovery of the dendritic cell and its role in adaptive immunity.

TLR4 is found in the membrane as a complex with myeloid differentiation protein 2 (MD2). Optimal binding with LPS to the TLR4-MD2 complex also requires the glycosyl phosphatidylinositol (GPI)-anchored, macrophage membrane protein CD14, and the LPS binding protein (LBP) which is an accessory protein that circulates freely in the blood (Jiang *et al.*, 2000).

Mice are unresponsive to LPS if any of TLR4 (Hoshino *et al.*, 1999), MD2 (Nagai *et al.*, 2002, Hamann *et al.*, 2004) or, CD14 (Haziot *et al.*, 1995) is deleted in the genome by homologous recombination. TLR4 has a large extra-cellular domain containing multiple leucine rich repeats (LRRs). Not surprisingly, evolution across species has led to variation in the extracellular domain of TLR4 and so species vary in their response to specific forms of LPS produced by different bacteria (Vaure & Liu, 2014).

There are two main divergent TLR signalling pathways with each TLR differentially recruiting the adaptor proteins: the MyD88 dependent signalling pathway and the TRIF/TRAM MyD88 independent pathway; only TLR4 utilises both these pathways (see Figure 1.3).

The MyD88 dependent pathway involves TIRAP which recruits MyD88 to the cell surface for TLRs 2 and 4, and to the endosomal membrane surface for TLR9 (Kagan & Medzhitov, 2006). Unlike TLR2 and TLR4, TLR9 activation does not depend upon TIRAP, whose actions reflect the phosphoinositide-binding domain to which lipids are targeted (Bonham *et al.*, 2014). Following ligation, MyD88 then binds with IRAK2, IRAK4 and then IRAK 1 producing a Myddosome, which is dependent upon molecular complementarity and surface electrostatics in order to be phosphorylated and activated (Kollewe *et al.*, 2004, Lin *et al.*,

2010). IRAK1 associates with TRAF6, UBC13 and UEV1A and together with TAK1,TAB1, TAB2 and TAB3 drives TAK1 activation which activates two pathways: the IKK complex-NFKB pathway and the MAPK pathway (Chen, 2012). NFKB induces proinflammatory gene expression, whilst MAPK signalling involves ERK1, ERK2, p38 and JNK which activate the AP1 family of transcription factors which bind FOS and JUN families of bZIP proteins which provide regulatory control.

The MyD88 independent/ TRIF dependent pathway involves TRAM recruiting TRIF. This complex then interacts with TRAF6 and TRAF3. TRAF 6 interacts with RIP1 which activates TAK1. Downstream NFKB and MAPKs, and TRAF3 recruit TBK1, IKKi and NEMO for IRF3 phophorylation which can then induce type I IFN genes (Akira *et al.*, 2006, Kawai & Akira, 2010, Kawasaki & Kawai, 2014).

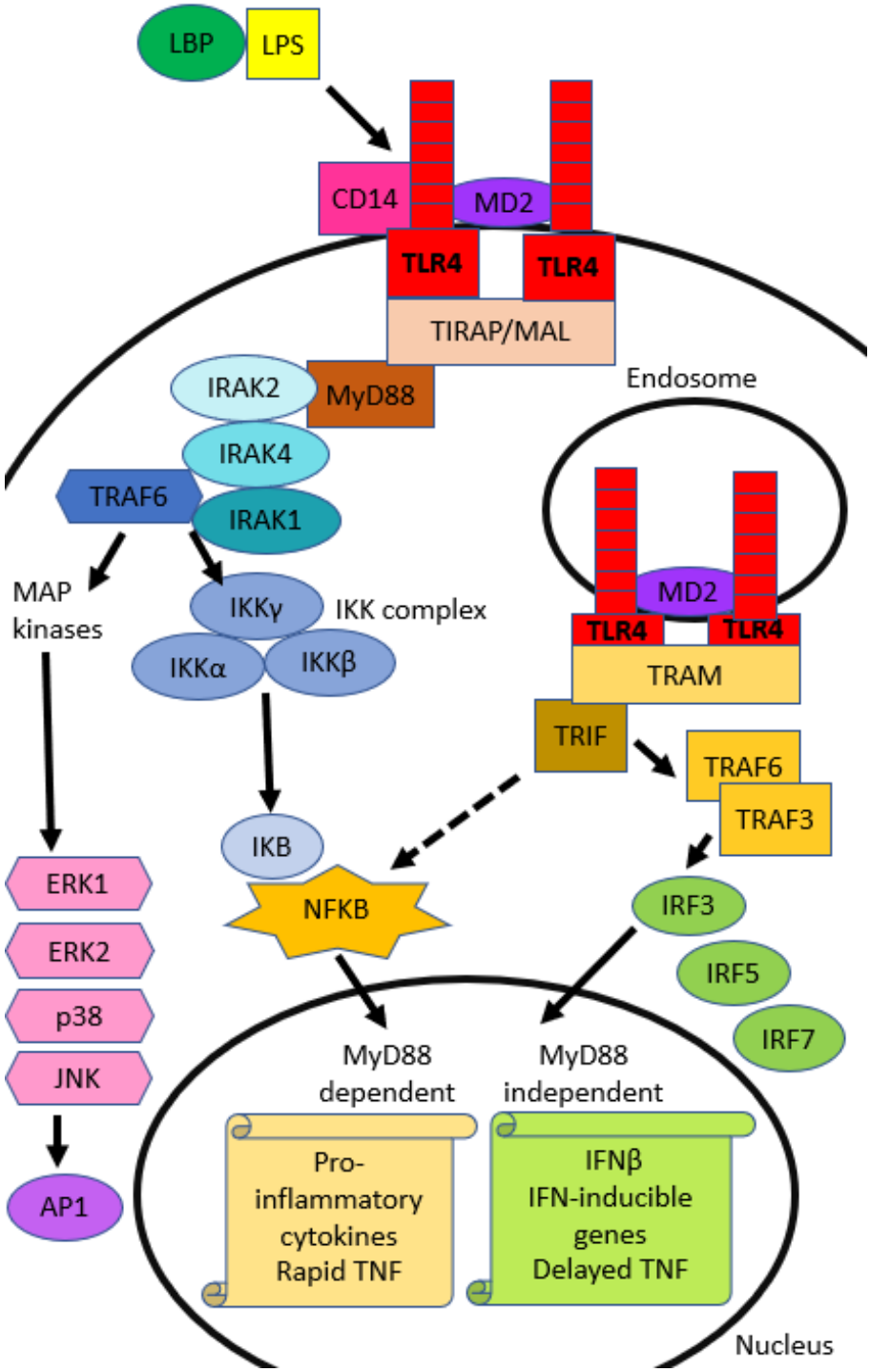


Figure 1.3 LPS signalling through TLR4
Adapted from (O'Neill & Bowie, 2007, Kawai & Akira, 2010, Kawasaki & Kawai, 2014). The MyD88-dependent pathway induces NFKB and the expression of pro-inflammatory cytokine genes. The MyD88-independent pathway induces NFKB (delayed) or IRF3, which leads to expression of IFN inducible genes.

Activation of macrophages by LPS involves the sequential induction and repression of thousands of genes, demanding tight regulation and including numerous feed-back control genes (induced early on in any response), which act at every level of the cascade (Adams, 1989, Nilsson *et al.*, 2006, Mosser & Edwards, 2008, Ghisletti *et al.*, 2010, Ostuni & Natoli, 2011, Beyer *et al.*, 2012, Raza *et al.*, 2014, Hume, 2015). This means that many overlapping pathways are utilised, requiring regulation over many regulatory elements such as transcription factors, as well as the expression of responder proteins involved in the cascade of activation (Natoli, 2010, Zanoni *et al.*, 2011).

Certain sets of genes share regulation through specific transcription factors to ensure the temporal response is appropriate for the common function that a specific set of genes is required to achieve, for example responding to interferon or genes required in early inflammation or endocytosis (Hume, 2015). Macrophages from different mouse strains have been found to vary in their transcriptional response to activation due to lineage-determining transcription factors influencing the epigenetic and transcriptional landscapes (Heinz *et al.*, 2013).

Transcription factors function by binding to regulatory regions sharing specific motifs, either proximal promotors or distal enhancers, and initiating transcription (Schmidl *et al.*, 2014). Macrophage enriched genes include transcription factors which are linked to likely binding sites in phagocyte-restricted promotors (Hume, 2015). Amongst the most -studied is the macrophage-specific transcription factor PU.1, which binds to purine-rich motifs repeated in the proximal promoters of most macrophage-expressed genes including *CSF1R* (Rojo *et al.*, 2017). Targetted mutation of the Spi -1 protooncogene gene, PU.1 gene (*SPI1*) in mice led to almost complete loss of myeloid differentiation (Scott *et al.*, 1994, McKercher *et al.*, 1996, R. McKercher *et al.*, 1999, Schulz *et al.*, 2012). Members of many other transcription factor families, notably AP1, CEBP, KLF, IRF, MAF and MITF families, interact with promotor and enhancer motifs in macrophage expressed genes (Rojo *et al.*, 2017). The most studied transcriptional activation event in the response to LPS is the translocation of the NFkB complex to the nucleus, but this is only part of a complex cascade of inducible transcription factors which includes AP1, IRF and other transcription factor families (Lawrence & Natoli, 2011).

Transcription factors mediate their actions in part by chromatin remodelling, as chromatin barriers can block transcription factor binding and therefore enable different programs of

expression, allowing separation in the temporal response between primary response genes (PRGs) and secondary response genes (SRGs) (Saccani *et al.*, 2001, Hargreaves *et al.*, 2009). Chromatin remodelling can be influenced by the CpG content (CpG islands) of the promotor region, which in turn affects the formation of a stable nucleosome (Ramirez-Carrozzi *et al.*, 2009). Other epigenetic influences over transcription include histone acetylation, trimethylation at lysine 4 of histone H3 (H3K4me3), DNA hypomethylation and the degree of sensitivity to DNase and RNA polymerase II (RNAPII) (Foster *et al.*, 2007, Hargreaves *et al.*, 2009, Ramirez-Carrozzi *et al.*, 2009).

1.6 Variation in innate response

1.6.1 Evidence for differential gene expression across species

Although the basic biology of macrophage signalling is conserved between species and individuals, fundamental differences in response to stimuli such as LPS have been well reported (Schneemann & Schoeden, 2007). Further evidence that species vary in their response to pathogens is reflected in the many infectious bacterial diseases which show species specificity in susceptibility. Ruminants are particularly sensitive to Clostridial disease, including *Clostridium chauvoei* (blackleg), *Clostridium septicum* (braxy), *Clostridium perfringens B* or *C* (dysentery/enterotoxemia) as well as other bacterial infections such as *Bacteroides melaningenicus* (foot rot), *Chlamydophila abortus /Chlamydiosis* (EAE abortion), *Campylobacter* (vibriosis), *Mycobacterium avium* subspecies *paratuberculosis*, and parasite infections such as *Toxoplasma gondii* (Toxoplasmosis), *Haemonchus contortus* (barber's pole worm) and *Eimeria* (coccidia).

Many previous studies in multiple species, have examined macrophage differentiation and activation in a genome wide study of gene expression (Ravasi *et al.*, 2007, Ramsey *et al.*, 2008, Fairbairn *et al.*, 2011, Kapetanovic *et al.*, 2012, Baillie *et al.*, 2017). The macrophage response to LPS has been found to be fundamentally different between mice and humans, with only 30% of orthologous genes being induced in both species (Schneemann & Schoeden, 2007, Schroder *et al.*, 2012). This will be discussed in more detail in later chapters of this thesis in the context of comparative analysis of the response of sheep macrophages. PRR themselves are differentially regulation between species; TLRs 2,3 and 4 have functional promotor differences between mice and humans (Heinz *et al.*, 2003, Heinz *et al.*, 2013). TLR9 is expressed by mouse macrophages, but not by human.

1.6.2 Variation in response between individuals

Individuals vary within any population (Darwin, 1859) and it has long been established that individuals within any population vary in their response to pathogens. Micororganisms exert strong selective pressure on genes of the innate immune system and antagonistic co-evolution between pathogen and host is a driving force of species molecular evolution (Paterson *et al.*, 2010). Macrophage activation involves the temporal induction of numerous feedback control genes which suppress inflammation and are known to be highly polymorphic within species and produce hyper-inflammatory phenotypes if individuals possess null mutations (Gilchrist *et al.*, 2006, Gilchrist *et al.*, 2008, Baillie *et al.*, 2017).

The complexities of inflammation in relation to the checkpoints and actions of specific genes and gene products underlying individual variation have been extensively reviewed (Nathan, 2002, Wells *et al.*, 2003, Wells *et al.*, 2005, Medzhitov, 2008). A recent large study of human monocyte responses to LPS (Fairfax *et al.*, 2014)demonstrated that as many as 80% of expressed genes show heritable variation in the level of expression between individuals (so-called eQTL).

The inflammatory response has to balance survival during infection and injury with the impact it has on tissue function, causing pathology (Medzhitov, 2010). The huge variation in susceptibility to pathogens demonstrates the functional genetic diversity of the immune response. Single gene (Mendelian) disorders are rare but can inform on the underlying mechanisms and protection against specific infections, an example includes heterozygosity for the sickle cell haemoglobin (HbS and HbC) which result from separate mutations in the β-globin gene, which provide protection against *Plasmodium falciparum* malaria because the environment of the sickle cell red blood cell is unfavourable to the parasite (Allison, 1954).

Another example of a single gene providing protection against pathogens is *natural resistance-associated macrophage protein 1* (*NRAMP1* or *SLC11A1*). NRAMP1 is a divalent metal transporter for iron, manganese and cobalt which is expressed in the late endosomal and phagolysosomal membranes, specifically in phagocytes (Gruenheid *et al.*, 1997, Forbes & Gros, 2003), and promotes efficient haemoglobin iron recycling (Biggs *et al.*, 2001). A coding variation in *NRAMP1* underlies the Bcq locus in mouse, which controls susceptibility to several intracellular pathogens such as *Salmonella, Leishmania* and *Mycobacterium* (Vidal *et al.*, 1993, Vidal *et al.*, 1995). By restricting the phagolysosome of iron, which is

required by the pathogens for growth (Forbes & Gros, 2001), the antimicrobial agent nitric oxide is synthesised (Fritsche *et al.*, 2007).

Polymorphisms in multiple genes surrounding TLR signalling are known to have implications for infectious diseases in humans (Miller *et al.*, 2005). Children with inherited IRAK4 deficiency were shown to not activate NFkB and MAPK in response to ligands of TIR bearing receptors, resulting in a failure to induce downstream cytokines and all developed pyogenic bacterial infections (Picard *et al.*, 2003).

Amino acid polymorphisms are uncommon in human TLR4, but have been found to cluster in the cytoplasmic domain so as to alter the sensitivity of detection and magnitude of the response (Smirnova *et al.*, 2000, Smirnova *et al.*, 2001, Miller *et al.*, 2005).

Mutations in the extracellular domain of TLR2 have been shown to impair mycobacterial signalling in mice, leaving them more susceptible to lepromatous leprosy and *Mycobacterium tuberculosis* (Bochud *et al.*, 2003).

Recognition of many of the genetic determinants of susceptibility has not yet moved into practical clinical treatment options, but as research, understanding and reports increase then realising the potential for developing novel vaccines and medical interventions become the most important goal for future studies (Burgner *et al.*, 2006).

1.7 Immunometabolism

Arachidonic acid metabolites such as prostacyclin (PGI2), thromboxane B2, prostaglandin E2, prostaglandin F2α, leukotriene C4 (slow reacting substance of anaphylaxis), and leukotriene B4, a potent mediator of chemotaxis have long been recognised as important in homeostasis and in the macrophage response with their release an important aspect of function (Scott *et al.*, 1982, Adams & Hamilton, 1984).

Regardless of the stimulus, macrophage activation and effector functions place demands on energy generation and cellular metabolism. Major metabolic changes have to take place for initiation of any immune response and many of the cytokines dictate endocrine regulation over whole body metabolism. The challenge is for immune cells to find adequate energy sources in a difficult environment. Immunometabolism relates to the intracellular metabolic changes and reprogramming that take place in response to immune stimuli, which is increasingly recognised as altering the function of immune cells and has been reviewed extensively by others (O'Neill *et al.*, 2016, Gaber *et al.*, 2017). Nutrients and

metabolites moderate inflammatory pathways as well as immune cell differentiation and when the ordered interactions between the immune response and metabolic response are disrupted a range of pathologies develops (Hotamisligil, 2017).

Given that the sheep has such different circulating metabolites to that of humans and mice, with distinctive hepatic gluconeogenic metabolism, it is anticipated that that ruminant macrophages will have distinct regulation of intermediary metabolism in immune cells.

This thesis will focus particularly on the transcriptome surrounding immunometabolism in the sheep and will be considered in more detail in chapter 5.4, where I will examine the expression of metabolism-associated genes in sheep macrophages to seek evidence for adaptation to such nutrient availability.

1.8 The relevance of sheep: Man's most ancient provider

1.8.1 Worldwide importance of sheep

Sheep were amongst the first domesticated livestock species, over 11,000 years ago (Larson *et al.*, 2014). This domestication has had a major impact on the modern sheep in comparison to their wild ancestor and like all domesticated animals, many of the "domestication syndrome" traits, first recognised by Darwin, can be identified, such as docility, endocrine changes, altered reproduction pattern and output, altered coat colour, facial neotony and a reduction in size.

Today sheep represent a major livestock species throughout the world, providing meat (mutton and lamb), milk, wool and skins. They are able to exploit pastures that are unsuitable for other agricultural purposes and can be integrated into other agricultural systems for efficient grassland management or crop rotation.

Cellulose is a complex and architecturally diverse macromolecule made of up of repeating glucose units (Klemm *et al.*, 2005). Mammals are unable to produce the enzymes required to access this energy source. The rumen evolved in some herbivorous mammals to support specialised fermentative microbes (bacteria, fungi and protozoa), to convert the lignocellulose-rich plant materials, into VFAs, methane and carbon dioxide. The ruminant animal co-evolved specialised energy and lipid metabolism to utilise the fermentative by-products as mentioned briefly above; vfas (acetic, propionic and butyric acid), the microbial protein (90% of amino acids reaching the small intestine) and vitamins provided by this specialised fermentative microbiome (Doreau & Ferlay, 1994).

Both ruminant (including their innate immune system gut barrier) and microbiome have evolved together over millions of years (Russell & Rychlik, 2001) and are inextricably linked, as they depend upon one another for survival—a classic example of mutualism (Flint *et al.*, 2008).

Within the UK there is a stratified system of sheep production that is unique and ideally suited to traditional sheep breeding management. Hillbreeds are adapted to life in inhospitable environments and are responsible for producing the replacement hill lambs, lightweight store or finishing lambs and cast ewes. They include breeds such as the Scottish Blackface, having desirable traits of increased growth rate, litter size and mothering ability. Upland areas then utilise the cross between the regular aged or cast hill ewe and the wool producing long wool rams such as the border Leicester. The resulting hybrid ewes are then crossed with terminal sire rams such as the Texel to produce the majority of UK finished lambs for meat. In the case of the Texel breed they possess a desirable mutation, which prevents translation of their *Myostatin* gene which is responsible for producing growth and differentiation factor 8 (GDF8). Skeletal muscle development is not then actively inhibited, resulting in double muscling and less carcass fat (Miar *et al.*, 2014). Down breeds are kept on low ground farms and produce finished lambs and replacement terminal sire rams. Variations of this system can also be used to produce slaughter lambs, such as mating draft hill ewes directly with terminal sires.

Heterosis generated by this management system provides significant improvement to the profitability of sheep farming with crossbred lambs shown to have improved survivability and to grow faster, crossbred ewes known to be more fertile and wean more pounds of lamb and crossbred males shown to be more fertile. It is this first cross animal (Scottish Blackface (BF) crossed with Texel (T)) that has been studied in this project.

1.8.2 Sheep Genome and Transcriptome

The International Sheep Genomics Consortium (ISGC) and United States Department of Agriculture National Institute of Food and Agriculture funded and produced the latest sheep genome assembly (OarV3.1) using male and female Texel animals and also provided transciptome information for 40 different tissues (Bahcall, 2014, Jiang *et al.*, 2014). This work highlighted sheep specific genetic changes surrounding lipid metabolism that result in changed skin barrier lipids and their involvement with wool synthesis, as well as noting the

increased role of volatile fatty acids in ruminants compared with nonruminant animals (Bahcall, 2014).

The BBSRC funded Sheep Atlas project at the Roslin Institute, to which my project contributed macrophage-related data, was the largest gene expression dataset of its kind, examining gene expression (RNA-Seq) data from tissues at every stage of development (blastocyst to adult) and across 120 tissues of both male and female adult sheep (Clark *et al.*, 2017). Knowing when and where a gene is expressed during the development of an animal, provides information regarding the specific biological role as well as improved understanding of functional sequence (Freeman *et al.*, 2012, Mabbott *et al.*, 2013, Hume & Freeman, 2014). This project provides functional information for many of the unannotated transcripts in the wider atlas through the 'guilt by association' principle (Oliver, 2000, Krupp *et al.*, 2012). The animals chosen were commercial cross-breeds between Texel, the breed chosen for the sheep reference genome (Bahcall, 2014) and the disparate Scottish Blackface, to maximise heterozygosity. All the animals were subjected to whole genome resequencing. Cross-bred animals were used to enable the identification of expressed single nucleotide variants (SNVs) that provide the basis for assessment of imbalanced expression of the two parent alleles. The use of the same atlas animals in the current project enables a comparison between the data obtained on macrophages *in vitro* with transcriptomic data from a wide range of primary cells and tissues. Furthermore, the data on stimulated macrophages generated herein makes a significant input to the wider atlas project. Using F1 crossbred animals also maximises the heterozygosity, so that the expressed genetic variation and allele specific expression (ASE) can be explored.

The Functional Annotation of Animal Genomes (FAANG) is 'a coordinated international action to accelerate genome to phenome' and recognises the need for improving functional annotation (Andersson *et al.*, 2015). The data from the Sheep Atlas, including the BMDM response to LPS samples generated in this project is freely available to support this community, as well as being deposited in the European Nucleotide Archive.

1.9 RNA-Seq technology and transcriptomics

Gene expression microarrays have provided much insight into the changes in transcriptional regulation that take place in macrophages in response to environmental stimuli (Martinez *et al.*, 2006). Next generation sequencing technology (first introduced in 2005) (Margulies *et al.*, 2005), has revolutionised characterisation of eukaryotic

transcriptomes, enabling novel gene clusters to be identified owing to increased transcript quality and quantity, with novel splice variants recognised and a larger dynamic range possible for detection (Wang *et al.*, 2009, Ozsolak & Milos, 2011).

There are two broad categories of RNAs, those which encode amino acids sequences and are translated for forming proteins and those which do not. Messenger RNAs (mRNAs) are translated into proteins and possess two structures which regulate their stability, polyadenylation (long runs of adenosine residues (polyA)) at the 3' end and the further esterified triphosphate residue at the 5' end that forms a structure called a cap.

The second category contains the non coding RNAs (ncRNAs) are involved in many nucleocytoplasmic functions. These include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), which both play essential roles in the maturation of mRNA (splicing) and rRNA (RNA modifications) within the nucleus, and microRNAs (miRNAs) which have been found to regulate transcription (Mattick, 2001, Memczak *et al.*, 2013, Zhang *et al.*, 2013, Wu *et al.*, 2014, Fang & Fullwood, 2016). A novel class discovered first with the cloning and sequencing of full length cDNAs is the mRNA-like, polyadenylated long non-coding RNAs (lncRNA) (Mattick, 2001). The functional annotation of the mammalian genome (FANTOM) project has been central to the discovery and identification of these transcripts (Carninci *et al.*, 2005). Most lncRNA are expressed at low levels, a few molecules per cell, and with a few exceptions, the functions of lncRNAs are unknown. Because of their low expression they are difficult to assemble reliably from short read RNA-Sequencing as shown in a very recent paper from The Sheep Atlas project which included sequence data from my project (Bush *et al.*, 2018).

Aim of the project

Sheep have evolved as a species under specific environmental pressures, which has resulted in particularly complex innate immune system biology surrounding the relationship with their complex fermentative microbiome. Sheep vary in their response to various pathogens in comparison to other mammals, including closely related ruminant species, and between individuals of the same flock. It is expected that sheep have a unique innate immune relationship with their microbiome and circulating products of fermentation and their macrophage transcriptional response to TLR4 activation.

The aim of this project was to define the transcriptome of sheep macrophages and their response to LPS using RNA-Seq technology. Two outcomes were anticipated (1) a major contribution to the annotation of the sheep transcriptome and genome since studies of other species have demonstrated that macrophages are amongst the richest and most diverse sources of novel transcripts (Wells *et al.*, 2003). (2) Comparative analysis of the sheep macrophage transcriptome with other animal species, enabling identification of ruminant-specific, or sheep-specific innate immune regulation.

Chapter 2 General Methods and approaches used to quantify transcript and gene expression

This chapter addresses the methods that were used to obtain differential transcript expression estimates for each sample used in my project. I was involved in all sample collections at Dryden Farm, assisting Tim King in the schedule 1 killing and then dissection and tissue harvesting. The immediate processing of the samples post euthanasia, back in the wet lab and the preparation of these samples for cryopreservation was performed by Dr Emily Clark, Dr Rachel Young, Ms Iseabail Farquhar, Dr Lucas Lefevre, Dr Clare Pridans, Dr Kristin Sauter, Dr Anna Raper and Ms Lyndsey Waddell. Dr Rachel Young and Dr Lucas Lefevre were responsible for the culture and RNA extraction of the monocyte derived macrophages. All the bone marrow samples were cultured, treated and had RNA extracted by myself and all the bioinformatics described in this project was done by myself, with guidance from my supervisor Dr James Prendergast. A brief outline of how RNA-Seq data was generated by Edinburgh Genomics is provided, before a detailed overview of the RNA-Seq processing, programmes and tools that were used to obtain transcript expression level estimates that are examined in subsequent chapters of this thesis.

2.1 Ethics statement

All samples for this project were collected post mortem. Euthanasia was performed by personnel licenced to perform schedule 1 procedures.

2.2 Animal Selection

The animals used in this project were six adult Scottish Blackface cross Texel animals, two disparate breeds in order to maximise heterozygosity for study, with the Texel breed having been utilised in producing the reference genome OarV3.1. Three females and three males were all approximately two years of age and in good health, bodily condition and with similar husbandry histories. Having been purchased for the study they were all kept on Dryden farm for at least 2 weeks prior to culling. Identical feeding and husbandry routines were implemented for each individual, ensuring that any differential environmental influences were minimal (Rosselot *et al.*, 2016). These animals formed part of an extensive transcriptional sheep atlas project in the Hume laboratory funded by the BBSRC and provided a wide range of other cells and tissues for transcriptional profiling. The first phase of this project was published in 2017 (Clark *et al.*, 2017).

My project has focussed specifically on the identification, characterisation and annotation of transcripts expressed and regulated in cells of the innate immune system, specifically on the activation of macrophages generated from bone marrow progenitors *in vitro*. Other immune cell types that were collected and utilised to a lesser extent in order to compare transcriptomic profiles, include macrophages derived by lavage of the lung (alveolar macrophages (AMs)), blood leucocytes (BLs), peripheral blood mononuclear cells (PBMCs which include circulating blood monocytes) and monocyte derived macrophages (MDMs).

All immune cell populations were prepared as for the previous smaller atlas project for the domestic pig (Freeman *et al.*, 2012) and the preparation of these cell types is discussed below. Other individuals and groups who were involved in the collection and processing are referred to and acknowledged accordingly.

2.3 Killing, tissue collection, cell isolation and cryopreservation.

All animals were killed by the schedule 1 cull method of electrocution followed by exsanguination. This method avoided the use of barbiturates for euthanasia which are known to cause significant tissue damage, irrespective of species, sex and agent used (Grieves *et al.*, 2008). All animals were killed at the same time of day (9.30am) which minimises the confounding variable of circadian rhythmicity between individuals (Keller *et al.*, 2009, Man *et al.*, 2016, Rosselot *et al.*, 2016).

I collected blood from each animal during exsanguination, into a jug containing Acidic Citrate Dextrose (ACD) to prevent clotting (10ml ACD/100ml blood) and immediately transferred it to 50ml falcon tubes to provide pure blood mononuclear cells, blood leukocytes and monocyte-derived macrophages from each animal (discussed in more detail below). All tissues were removed by a team of veterinarians including myself, within 1hr 30mins of culling, and cells immediately harvested and processed as quickly as possible, so as to minimise post mortem effects on RNA levels.

2.3.1 Bone Marrow Processing

The protocol originally developed for pigs (Kapetanovic *et al.*, 2012) was used to prepare bone marrow cells from five posterior ribs extracted from both sides of each animal, ensuring both ends of each rib remained closed so as to avoid contamination. They were immediately placed in phosphate buffered saline (PBS) within a sterile plastic bag and transported on ice back to the laboratory for processing inside the microbiological safety

cabinet. The bones were stripped of all intercostal muscle and cleaned using ethanol, cut into Sections and flushed from both ends with RPMI-1640 (Sigma UK), containing 5 mM EDTA to prevent clotting. This marrow cell suspension was then filtered through a cell strainer and centrifuged at 400g for 5 mins. The supernatant was removed and the cell pellet resuspended in 5ml erythrocyte cell lysis buffer (10mM $KHCO_3$, 150mM NH_4Cl, 0.1mM EDTA pH 8) for 5 mins, after which RPMI-1640 media was added to dilute the buffer and the cells were spun again at 400g for 5 mins. The supernatant was removed and the cell pellet resuspended using sheep medium (400ml RPMI1640 (Sigma UK),100ml heat inactivated sheep serum (Sigma UK), 5ml Glutamax (Invitrogen UK) and 0.5ml Penicillin/Streptomycin (25U/25µg/ml, Gibco UK)). The cells were counted and then aliquoted in cryovials at approximately $5x10^7$cells/ml freezing medium (80% sheep serum and 20% DMSO), adding the freezing medium slowly to avoid shocking the cells with DMSO. The labelled cryovials were then placed in an isopropanol freezing unit and placed in -80^{o}C freezer for 24hrs and then transferred to long term storage at -155^{o}C.

2.3.2 Processing of blood cells

Total blood leukocytes were isolated by the Hopkins laboratory using published methods (Montgomery & Sise, 1990). Peripheral blood mononuclear cells (PBMCs) were isolated as previously described for the pig (Fairbairn *et al.*, 2013) except that 2% serum was used throughout. To isolate monocytes by adherence, PBMCs were then cultured overnight in complete RPMI + recombinant human CSF1 (rhCSF1; a gift from Chiron, Emeryvillle, CA) in a 100cm^2 Sterilin plate at 10^6 cells/ml. The following day the supernatant was removed and TRIzol ® Reagent (Invitrogen, Darmstadt, Germany) was added to the cells. The cell lysate was then aliquoted and frozen in cryovials at -80^{0}C. The remaining PBMCs were frozen in cryovials at a concentration of 1 x 10^8/ml in 90%FCS/10%DMSO or cultured for 7 days in complete medium in the presence of rhCSF1 to produce the monocyte derived macrophage samples (MDMs) (discussed further in Section 2.3.5).

2.3.3 Alveolar Macrophage Isolation

Briefly, the chest was opened and trachea double clamped and the entire pluck was removed. Once exteriorized, the heart and lungs were separated and with the tracheal clamp still in place, the lungs were transported on ice back to a microbiological safety cabinet for processing. The lungs were lavaged twice with 1 litre of PBS, and cells recovered by centrifugation.

If necessary, red blood cells were lysed by resuspending the pellet in red blood cell lysis buffer (NH_4Cl, $NaHCO_3$, EDTA). Cells were plated out at 2 x 10^6 cells/ well in a tissue culture plastic 6 well plate overnight in 2ml sheep media with rhCSF1 (see below). The following day, supernatant was removed and replaced with 1ml TRIzol®, then contents transferred to a 1ml cryovial for storage at -80 in preparation for RNA extraction.

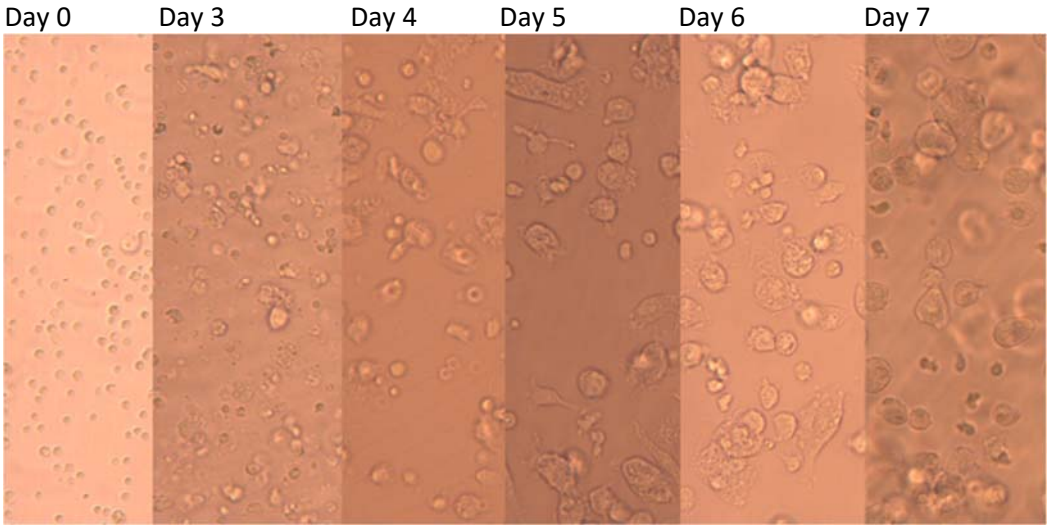
2.4 Cell culture of Bone Marrow Derived Macrophages and LPS treatment

For the generation of bone marrow-derived macrophages (BMDMs), cells were initially recovered from long term storage at -155°C by thawing each vial rapidly in the water bath (37°C) for 2 mins. The cryovial contents were transferred to a 15ml falcon tube and very slowly (so as to avoid the shock of sudden dilution) 9ml of sheep medium was added. The tubes were gently inverted a few times and then spun for 5mins at 400g. The supernatant was removed and the cells were counted and plated out onto 100mm^2 bacteriological plastic plates at 2.5x10^7 cells/plate in a total of 20ml sheep media + 200µl of recombinant human CSF1 (rhCSF1) to provide a final concentration of 10^4 U/ml (100ng/ml).

Plates were incubated at 37°C, inside an incubator (37 °C, 5% CO_2 in air). After 4 days, 10mls of fresh sheep medium + 100µl rhCSF1 were added. The cells were checked every 2-3 days and showed increased size, granularity, vacuolar appearance and adherence to the bacteriological plastic (Wang *et al.*, 2013). Figure 2.1 demonstrates the phenotypic changes that were visible in all cultured BMDM samples used in this project.

At the end of day 6 all cells, including the predominating confluent adherent monolayer, were removed by gentle scraping and flushing the plate with medium using a blunt needle and syringe. The cell suspension was centrifuged at 400g for 5 mins. Cells were counted and replated in a 6 well plate at 2 x 10^6 cells/well and a total of 2mls of sheep medium + rhCSF1 (100ng/ml). On day 7 the LPS time course commenced.

Figure 2.1: Morphological differentiation of sheep bone marrow cells in response to CSF1. Sheep bone marrow cells were cultivated in sheep medium with rhCSF1 and the plates were photographed (20 X magnification) on days 0,3,4,5,6 and 7 (images from left to right). The images are typical of the time course in each of the 6 sheep studied.



2.5 LPS Stimulation, cell photography and Trizol reagent treatment

The cells were left overnight in the 37^0C incubator to adhere to the tissue culture plastic. The five time points chosen for analysis, (0, 2, 4, 7, 24 hrs) were based upon the known temporal profile of the response to LPS in other species, including mice, humans, pigs and horses. (Kapetanovic *et al.*, 2012, Schroder *et al.*, 2012, Raza *et al.*, 2014, Karagianni *et al.*, 2016, Baillie *et al.*, 2017). LPS was added sequentially for each time starting with the longest incubation (24hrs) so that all the time points could be harvested and processed together. To initiate the LPS response, medium was replaced with 2ml sheep medium containing LPS from *Salmonella enterica* serotype Minnesota Re 595 to ensure a final concentration of 100ng/million cells, as used in pigs (Kapetanovic *et al.*, 2012). For every 2ml well, a further aliquot of rhCSF1 (100ng/ml) was added to ensure that the cells were saturated with the growth factor and that most BMDMs were in the cell cycle (Raza *et al.*, 2014).

At the end of the incubation, cells at the five time points were examined using light microscopy to determine whether LPS had induced the anticipated spreading on the substratum (Wenzel *et al.*, 2011). Every well was examined under the light microscope and photographed, before the medium was removed and replaced with 1ml TRIzol ®/well. Each

plate containing Trizol was gently agitated and the individual well/LPS time point sample contents transferred to a cryovial and stored at -80 °C.

2.6 RNA extraction, quality and quantification checks prior to submission for RNA-Sequencing at Edinburgh Genomics

In preparation for RNA extractions, all surfaces and devices to be utilized were thoroughly cleansed using RNase-Zap® (Ambion™, ThermoFisher Scientific UK) to remove all traces of ambient RNases and nucleic acid contaminants. Only RNase free reagents and consumables were used. The sample was thawed to room temperature for 5 minutes and 200µl BCP (bromochloropropane) (Sigma Aldrich) was added. The sample was left to stand for 3 minutes and during this time the homogenate separated into a clear upper aqueous layer (containing RNA), and interphase and red lower organic layers (containing the DNA and proteins). The sample was then centrifuged for 15 minutes at 12,000 x g, at 4°C. After centrifugation the upper aqueous phase was removed and RNeasy® Mini Kits (Qiagen UK) were used to extract total RNA from each sample. This method is widely used and reported to extract high yields and good quality RNA, avoiding the need to precipitate the RNA (Grabmuller *et al.*, 2015). The exact protocol specified in the manufacturer's instructions was followed for each extraction and also involved an RNase-Free DNase treatment set (Qiagen UK), again following the manufacturer's instructions.

The quantity and quality of RNA extracted for each sample was estimated using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington DE USA) and the RNA integrity estimated on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara CA USA).

2.7 RNA sample submission

RNA samples with the best RNA Integrity number equivalent (RINe) values were selected (all with RINe >9), from each individual at each respective time point and were submitted to Edinburgh Genomics as a single submission (see Appendix 2.1) and run on the Illumina HiSeq 2500 sequencing platform.

Samples from all individuals at all time points were sent for 125 bp, paired end sequencing at 25 million read depth using Poly A selected library preparation. In addition, the same 0 and 7 hr time point samples were sent for sequencing at 100 million read depth using ribodepleted total RNA library preparation.

2.7.1 Illumina TruSeq protocols for Ribodepleted (total) stranded RNA Library Preparation and PolyA-selected (mRNA) stranded Library Preparation used by Edinburgh Genomics.

RNA samples were subjected to either mRNA-Seq (>25M read depth, as done for the majority of Sheep Atlas samples, where the priority is to examine protein coding transcripts) or total RNA-Seq (>100M read depth, as done for chosen Sheep Atlas samples of particular interest) in order to balance costs, coverage of samples collected and read depth to enable exploration of the rarer transcript. The two protocols are described briefly here, since they influence the interpretation and analysis of the RNA-Seq data. All procedures were performed by Edinburgh Genomics.

The total RNA-Seq libraries were prepared using the Illumina Truseq total stranded RNA library preparation kit with (human/mouse/rat) Ribo-zero for the depletion of rRNA in the samples (Ribodepleted_totalRNA_ILLUMINA, 2013). The mRNA-Sequencing libraries were prepared using the Illumina Truseq stranded mRNA library preparation kit.(PolyA_mRNA_ILLUMINA, 2013).

The two library preparation kits differ in the pre-treatment of the RNA prior to cDNA preparation. For the ribodepleted total RNA library, the first step involved removal of both cytoplasmic and mitochondrial ribosomal RNA (rRNA), using biotinylated, target-specific oligos combined with Ribo-zero rRNA removal beads.

The first step for the polyA-selected mRNA library used oligo-dT-coated magnetic beads to purify the polyA containing mRNA molecules.

The RNA from each library prep was then fragmented using divalent cations at high temperature. The cleaved fragments were copied into first strand cDNA using reverse transcriptase and random primers. The first strand synthesis mix also included Actinomycin D which prevents spurious DNA dependent (but not RNA dependent) DNA synthesis (Ruprecht *et al.*, 1973). This prevents artefact second-strand cDNA which would produce false antisense transcription (Perocchi *et al.*, 2007).

The second strand was synthesised using DNA Polymerase I and RNAse H (which degrades the RNA strand of the heteroduplex), with dUTP replacing dTTP in this second strand marking mix. This means the strands can be identified from one another, as the polymerase used in the assay (a Uracil-DNA-Glycosylase(UDG)) selectively degrades the

strand containing the dUTP, leaving the remaining strand to be amplified from which to generate the cDNA library for sequencing (Borodina *et al.*, 2011). The cDNA fragments had a single 'A' base added and the adapter sequence was ligated. The enzymes and oligonucleotides required are all included in the Illumina Genomic DNA Sample Prep Kit that Edinburgh Genomics used.

After this, the products were purified using a Qiagen PCR Purification Kit and enriched with PCR. Both the TruSeq stranded Total RNA and mRNA HT Sample Prep Kits used by Edinburgh Genomics utilise a 96 well plate format with 96 unique adapter combinations, to index the libraries either manually (as was used for all the PolyA mRNA library samples) or using an automated robot (as was used for all the ribodepleted total RNA library samples).

2.8 Quality control checks on raw reads (Fastqc tables)

Raw read files of high throughput sequence data were returned by Edinburgh Genomics. All subsequent processing and informatics analysis was performed by myself. The fastq files were quality checked using the programme FastQC (Andrews, 2010) in Linux. This provided a modular set of analyses to ascertain whether there were any problems or biases within the data that needed to be addressed before further analysis. The per base sequence quality was excellent for all samples and no systematic issues were identified. Results including the per sequence quality scores which report if a subset of sequences have universally low quality scores, the per base N content which indicates if the sequencer has been unable to make a base call with sufficient confidence and the sequence length distribution which indicates whether there is varying lengths of sequence fragments, were all within acceptable limits and therefore no concern was felt about the quality of the results. Occasional failures in the per base sequence content, expected since the RNA-Seq libraries were being produced by priming using random hexamers, do not adversely affect the downstream analysis and so were ignored (Andrews, 2010). The per sequence GC content was similar for each sample. Failures in the sequence duplication levels, can be explained due to the nature of this RNA-Seq experiment in which some genes have exceptionally high abundance levels.

No more than 20% of the reads showed any adaptor content after 107bp. The reads were all 125bp in length. Accordingly, no adapter trimming was undertaken, as adapter sequences will only occur at the 3' end of the read and the local alignment soft clipping tool used in Bowtie2 will resolve this problem for those 20% of reads (see Fig 2.2). The

sequencing of small RNAs which would require adaptor trimming was not the priority for this project and the current coverage of the reference genome means that there is still a chance that although adaptors are supposedly synthetic and not to occur in the genomic sequence, there is a chance that they may appear in the sheep but have not yet been recognised.

2.9 Pipelines used to quantify transcript and gene expression (StringTie and Kallisto2) and BAMstat

The RNA-Seq data generated for each sample are required to generate transcript expression estimates: both for transcripts already identified in OarV3.1 (Aken *et al.*, 2016) and for those which have not yet been recognised (novel transcripts and gene models). This necessitated two different pipelines, Kallisto2 (Kallisto) and StringTie to maximise the strengths and ability of each. Kallisto2 (Bray *et al.*, 2016) utilised the reference transcriptome OarV3.1.81. StringTie (Pertea *et al.*, 2015) utilised the reference genome OarV3.1.81 (http://www.ensembl.org). The relative merits and outcomes of these pipelines are discussed in detail in chapter 3.

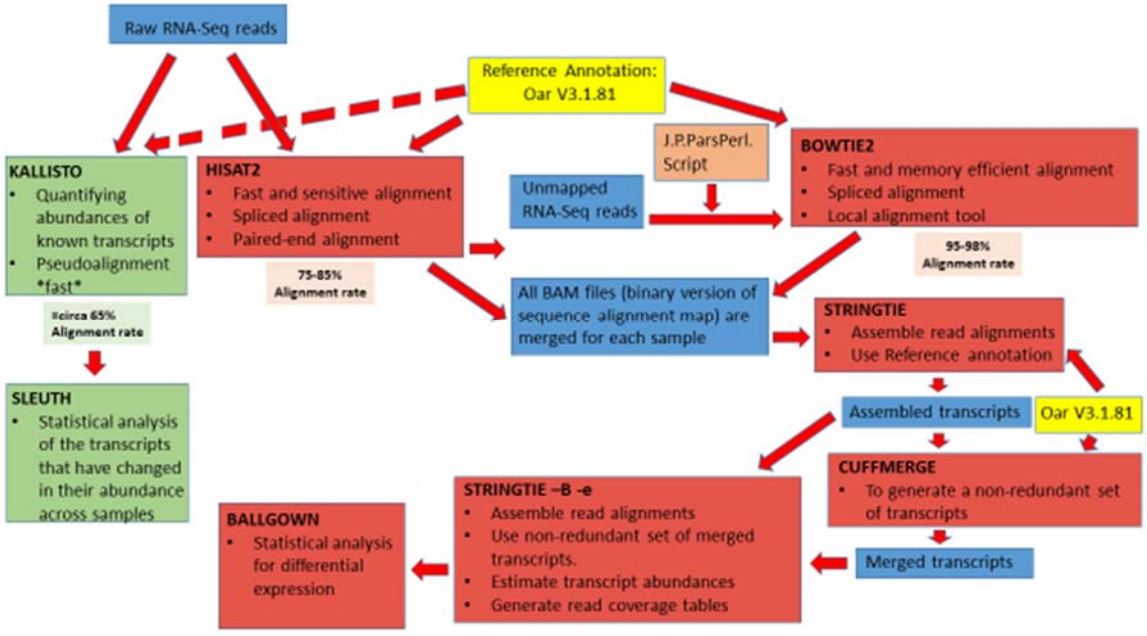


Figure 2.2: The two pipelines used to quantify transcript and gene expression for samples utilised in this project.

2.9.1 Kallisto Pipeline for processing raw reads

Kallisto2 (Pimentel *et al.*, 2017) utilises a method of pseudoalignment to match reads to a reference transcriptome (containing 28,757 transcript sequences for OarV3.1.81) of known coding and non-coding transcript sequences. The reference transcriptome was produced from the Ensembl 81 FTP download site using the coding and non-coding cDNA reference FASTA files for OarV3.1.81 (Bray *et al.*, 2016). The algorithm divides each read sequence into kmers of 31bp in length and allocates each kmer to a best match transcript. Because the read alignment does not include genomic mapping, time and computing power are saved and it is robust to errors in the reads (Bray *et al.*, 2016). The limitation is that the potential number of transcripts identified is constrained by the completeness of the reference transcriptome.

The programme Sleuth (Pimentel *et al.*, 2017) was used to estimate the confidence of differential expression from the gene expression estimates derived from Kallisto2. Sleuth uses statistical algorithms for differential analysis that leverage the bootstrap estimates of Kallisto2 (Pimentel *et al.*, 2017). The bootstrap provides a measure of the accuracy of the quantification of expression of a transcript by random resampling with replacement to generate estimates for the technical variance. For all samples the number of bootstraps was specified to be 1000.

The P values and q values obtained from Sleuth (discussed in more detail in Section 3.1.4) are a result of comparing the expression estimates of samples using a Wald test which tests the null hypothesis that the covariate for a given transcript is 0.

2.9.2 StringTie Pipeline for processing raw reads

After ensuring the quality of the RNA-Seq reads using FastQC, the raw reads were aligned to the reference genome (OarV3.1.81) using the programme HISAT2 (Pertea *et al.*, 2016). The known-splicesite-infile mode was utilised which takes a list of known splice sites which HISAT2 used to align reads using 'small anchors' (the python script that the manual provided was used to make this splice site index from OarV3.1.81). When HISAT2 was unable to make a valid alignment, the set of reads was passed to an UNMAPPED file, which would then be parsed. Parsing was carried out using a perl script provided by Dr James Prendergast which fixed a bug within HISAT2 that produces double copies of .1 (first strand) reads in the unmapped output file.

To recover and analyse the unmapped HISAT2 reads, Bowtie2(Langmead & Salzberg, 2012) was utilised in the local alignment mode. Local alignment meant that the ends of the read could be omitted or "soft trimmed". This meant that all the multi-mappers and reads that may have had some adapter contamination present were 'rescued', as well as allowing reads to be mapped where there is a problem in the reference, with this second chance to be mapped by Bowtie2. The Bowtie2 default is to find a valid alignment and continue to look for alignments that are better until the limit placed on the search effort is reached (the default for this project was a maximum of 20 dynamic processing problems failing in a row). When equally good choices are found, the program uses a pseudo-random number to choose the location. This pseudo-random number generator is re-initialized for every read using a "seed" which is a function of the read name, nucleotide string and quality string, so that the mapping results are reproducible. This project selected the "--very-sensitive" option in the command line. The wide area covering the speed/sensitivity/accuracy trade off space meant that the aligner ran more slowly in this default option but was more accurate and sensitive in deciding upon the optimal alignment. This default set the maximum number of "re-seeds" when attempting to align a read with repetitive seeds as 3 and the number of mismatches permitted per seed to be 0. The trade-off between speed and sensitivity/accuracy can be adjusted by changing the seed length, the interval between extracted seeds and the number of mismatches permitted per seed. Values used here were all default for prioritising accuracy over speed.

The two alignment files from each of these tools (HISAT2 mapped.bam and Bowtie2 mapped.bam) were then merged together to produce a single merged sequence alignment map (sam) file for each sample using SAMtools 1.2 and then sorted by reference position(Li *et al.*, 2009). SAMtools 1.2 was also used to generate an index file for each of these merged, sorted bam files.

The programme StringTie assembled these RNA-Seq alignments for each sample into potential transcripts, using a network flow algorithm as well as de novo assembly, to assemble and quantify full length transcripts representing multiple splice variants for each gene locus (Pertea *et al.*, 2015, Pertea *et al.*, 2016). The reference annotation file from OarV3.1.81 was also included to see whether the reference transcripts were found in the RNA-Seq data, computing the coverage of any that were recognised.

At the start of this project, the most up to date version of StringTie was version 1.0.4 which required that the programme Cufflinks was utilised as it includes a script called cuffmerge that was able to take all the assemblies for each animal and merge them together to generate a non-redundant set of transcripts (Trapnell *et al.*, 2010, Roberts *et al.*, 2011). This merged transcript gene transfer format (GTF) file was then used to repeat StringTie to estimate the transcript abundances for each individual and enable a comparison between individuals and samples. Subsequently, it was recognised that this pipeline has a bug. For a small minority of transcripts that significantly overlapped each other, Cufflinks assigned a unique ID and when StringTie was then performed for the second time, these transcripts were not recognised by StringTie and were automatically assigned an expression value of 0. This issue was recognised because a key known macrophage-expressed gene, *CSF1R* (discussed in Introduction and Chapter 4), was assigned a unique Cufflinks ID and accordingly appeared to be absent from the StringTie outcomes. The issue arose as *CSF1R (ENSOART00000006925)* has an upstream promoter within the 3' UTR of the neighbouring *PDGFRB* gene (Visvader & Verma, 1989), so expression from the two loci was merged and allocated a unique ID.

The more recent version of StringTie was 1.3.3, for which the need to rely on Cufflinks had been resolved and Pertea et al (2017) had added their own '–merge' command to achieve what previously was not possible. In repeating the pipeline in its entirety, using StringTie 1.3.3, *CSF1R* was found to be expressed at high levels at 0hr LPS as expected and down-regulated by LPS. The Ensembl transcript ID for *CSF1R* was still assigned to have an expression of 0 FPKM, as StringTie included this upstream promotor and allocated the expression to this novel transcript model (MSTRG.26740), which has an extra exon (22 exons rather than 21) compared to the reference.

From these read coverage tables that have been generated, the Ballgown software (Frazee *et al.*, 2015, Fu J, 2016) was used to identify the differentially expressed genes and transcripts between the different samples using linear-model based analyses.

2.10 Visualisation tools (Miru, R, IGV)

Various programmes and tools have been utilised to visualise the dataset and compare gene and transcript levels between individuals, such as R and R studio, which is a language and environment for statistical computing in order to create principal component and scatter plots, as well as the powerful visualisation tools Miru (http://kajeka.com) that

enable expression pattern recognition to create meaningful clusters. The Integrative Genomics Viewer (IGV, http://software.broadinstitute.org/software/igv/) was also used.

Miru (Kajeka, UK) calculates similarity matrices based upon pairwise correlation coefficients from expression estimates for each transcript or gene across all the samples and then renders these as networks in a 3D interactive environment. Many other large gene expression analyses have used the predecessor program BioLayout Express^{3D} (Theocharidis *et al.*, 2009, Mabbott *et al.*, 2011, Freeman *et al.*, 2012, Hume *et al.*, 2013). Clustering of co-expressed genes in this programme uses the Markov Cluster Algorithm (MCL) (Enright *et al.*, 2002). MCL works by examining the paths (multiple pairwise comparisons between the nodes) by taking random walks and determining the probability of 'walking' from one cluster/node to another using two operators, Expansion and Inflation (Theocharidis *et al.*, 2009). Clusters of genes that share transcriptional profiles tend also to participate in common pathways(Freeman *et al.*, 2012). This means that possible function of an unknown transcript can be inferred by examining the other members of the same cluster that do have a functional annotation.

The Integrative Genomics Viewer (IGV) enables visual, interactive exploration of large, integrated genomic datasets (Robinson *et al.*, 2011, Thorvaldsdottir *et al.*, 2013) and has been used to observe the coverage of reads after mapping with HISAT2 and Bowtie2 to the reference Oar V3.1.81 and highlight differences between samples, individuals and the reference models. IGV was used to create Sashimi plots for selected genes and genomic regions. This enabled quantitative visualisation of the splice junctions and comparison of differentially spliced exons and alternative transcript models across multiple samples, helping identify those that are not currently recognised in the reference OarV3.1.81 (Katz *et al.*, 2015).

2.11 Methods and tools used to functionally annotate key genes of interest.

Biomart (http://www.ensembl.org/biomart/martview/) was used to add known information for every expressed reference transcript, such as the associated gene stable ID, gene name, chromosome/scaffold name, strand, coordinates, gene type and % GC content (Smedley *et al.*, 2015).

Many of the biologically significant transcripts of interest identified had no functional annotation. These include OarV3.1.81 reference transcripts that lack any functional

annotation and are just identified by the Ensembl ID and those transcripts and genes that have been identified by the StringTie pipeline and are totally novel.

A variety of resources was utilised to assist in functional annotation of those transcripts/genes of interest which were unknown and lacked functional annotation, prioritising those that were found to be macrophage specific (within the sheep atlas project clustering analysis) or which demonstrated regulation and differential expression in the BMDM LPS response time course.

2.11.1 Annotating reference transcripts with no functional annotation.

All coding and non-coding reference transcripts were used for the Kallisto2 index. For those reference transcripts with no functional annotation, initially Ensembl was used to visualise the transcript alignment to the genome and the gene model (Aken *et al.*, 2016). For each protein coding transcript Ensembl provides a list of the nearest orthologues in other species. These are generated by Ensembl utilising phylogenetic trees generated by TreeBeST (Vilella *et al.*, 2009) which represent the evolutionary history of gene families. The degree of homology at the nucleotide level can be calculated and forms the basis for the description of orthologue relationship, be it one to one, one to many, many to many or between species paralogues (Jensen, 2001). Initially any 1:1 orthologues were examined. The initial priority was to note any that appeared within the same phylogenetic clade as the sheep, the Laurasiatheria, paying attention to the best target % id and query % id. Many of these genomes are lacking in functional annotation, similar to the sheep, so other species sets were examined, with particular attention to humans and mice in the knowledge that these are the most complete and annotated reference genomes.

To reinforce predicted orthology, the synteny of the genomic region was compared within Ensembl using the species comparative viewer. The molecular clock rates of evolution vary among organisms and are known to depend upon other biological characteristics that are also undergoing evolution, for example substitution rates are influenced by metabolism (Rand, 1994). Related organisms often show similar blocks of genes in the same relative positions within the genome, and shared synteny is critical for determining the orthology of genomic regions for different species. Each reference genome is functionally annotated to a different degree. By comparing other farm animals such as pig and cattle as well as genomes with better annotation such as human and mouse it was possible to add

confidence to proposed orthology relationships and to assign proposed annotation across species.

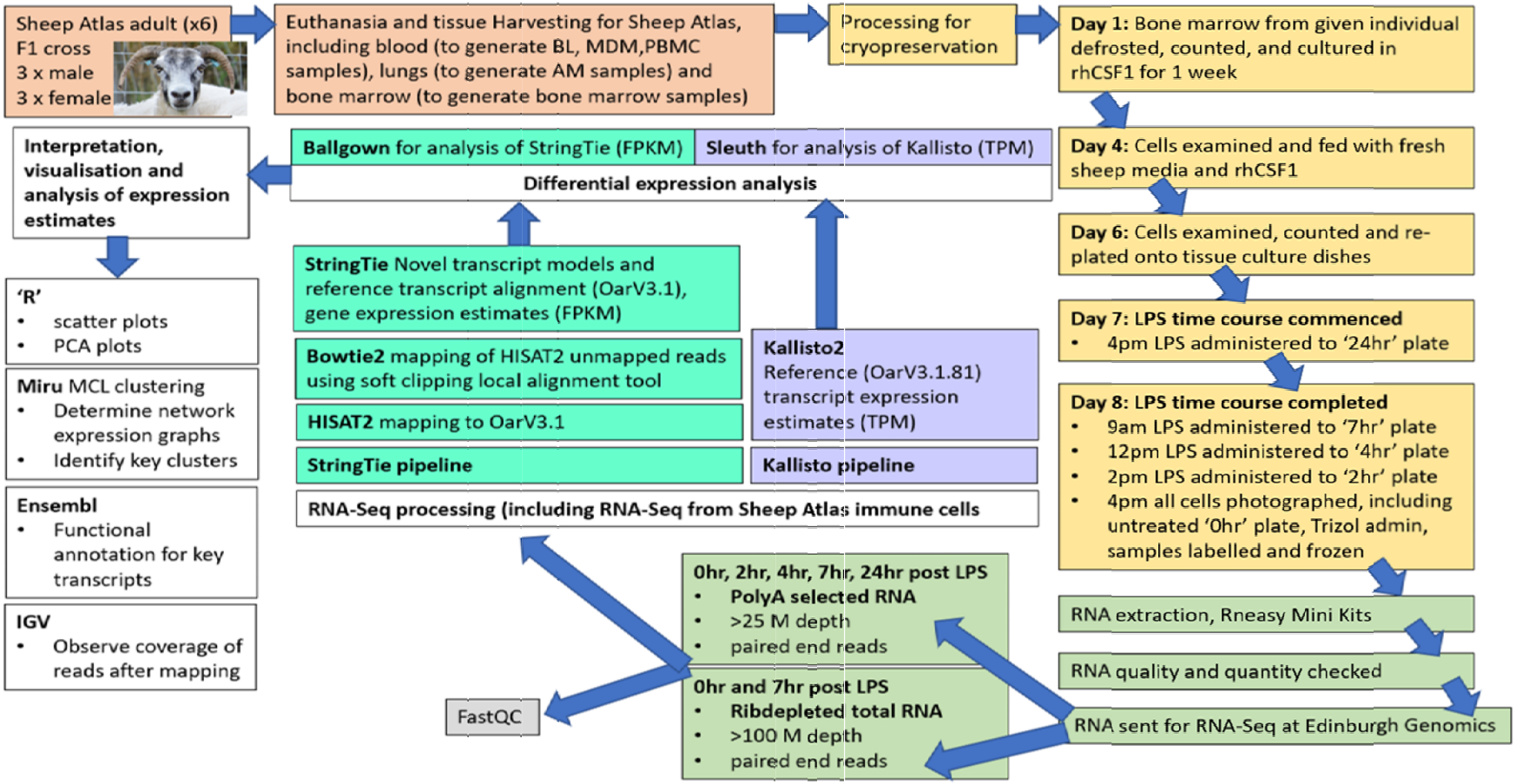
When genes had no nearest orthologues, Ensembl provided information about the transcript such as the domains and features recognised and any variations. The BLAST tool was used to determine or infer functional and evolutionary relationships that may be shared with other genes or gene families within the sheep genome or other species and calculate the statistical significance of any matches made (McGinnis & Madden, 2004, Ye *et al.*, 2006).

The reference transcript position and model was validated against relevant merged.BAM files (aligned using HISAT2 and Bowtie2) using IGV (Thorvaldsdottir *et al.*, 2013), to validate reference transcript models as well as highlight novel ones of potential future interest.

2.12 Summary of Chapter 2

This thesis uses cell culture to examine the transcriptional response of sheep macrophages to stimulation with lipopolysaccharide. High-throughput sequencing and advanced bioinformatics tools were then applied to analyse the resulting data. This chapter describes those methods that were applied in all experimental procedures, summarised in Figure 2.3. The subsequent chapters present the results and interpretation of the resulting data.

Figure 2.3 An overview of the experimental design, the biological sample sources, the data generated and the bioinformatics utilised for downstream interpretation in this thesis.



Chapter 3 Evaluation of Transcriptome Analysis Methods

3.1 Introduction

Once a draft genome is completed, the next focus is to identify the set of transcripts (the transcriptome) that are encoded and the ways in which genes and transcripts are utilised in any given cell type/tissue at a given developmental stage and under certain physiological conditions (Ekblom & Wolf, 2014, Conesa *et al.*, 2016). Although there are multiple steps that determine the functional output of the genome; including RNA processing, translation and post translational processing and modifications, the first level of control is transcription. Functional annotation of the genome starts with identification of the sequence and relative abundance of transcripts expressed in the widest possible range of cell types and tissues.

Transcriptomic technologies to analyse differential gene expression have evolved rapidly over the past 30 years. Differential display (DD) and differential hybridisation (Liang & Pardee, 1992), subtraction of cDNA libraries (Hedrick *et al.*, 1984), suppression subtractive hybridisation (SSH) (Diatchenko *et al.*, 1996), and serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), all managed to identify differential gene expression. However, these methods had low reproducibility and low efficiency for investigating the complexity of transcriptomes (Frolov *et al.*, 2003).

Microarray technology, when used for gene expression analysis, is based upon the hybridisation of a probe (cDNA or synthetic oligonucleotide). This probe is complementary to a known gene or gene fragment (immobilised on a solid substrate), to a sample of labelled cDNA which is copied from the RNA of a sample of interest (Lipshutz *et al.*, 1999, Baldi & Hatfield, 2002, Miller & Tang, 2009). They have been used extensively to determine the gene expression profiles of cell and tissue samples, including, relevant to this project, BMDMs from the pig (Kapetanovic *et al.*, 2012), and mouse (Schroder *et al.*, 2012), human MDMs (Martinez *et al.*, 2006) and bovine MDMs (MacHugh *et al.*, 2012). Microarray data has revealed many key signatures associated with the differentiation and activation of macrophages. The consistency of formats, notably the Affymetrix platform, has enabled meta-analysis and aggregation of data from multiple laboratories (Mabbott *et al.*, 2010, Kapetanovic *et al.*, 2013, Mabbott *et al.*, 2013, Regan *et al.*, 2018). Much of the documented evidence of individual and species variation in differential expression

estimates which this study utilises, to enable comparison of species, was derived using microarrays.

Microarrays do have some inherent technical problems, such as cross-hybridisation, non-specific hybridisation and a limit to the range of detection of any given probe/target, as they cannot provide the absolute detection of very highly expressed genes or the sensitivity to detect rarely expressed genes (Draghici *et al.*, 2006). RNA-Seq, does not rely upon any pre-determined sequence and there is no saturation limit, which means that genes with the greatest differential expression can be detected. With sufficient depth of sequencing, RNA-Seq can be superior in sensitivity for detecting low abundance genes/transcripts, and because it is not restrained by the number of target DNA sequences, it is able to detect a much greater variety of transcripts and splice variants.

RNA-Seq technology has largely superseded microarray analysis as it directly sequences RNA using high-throughput sequencing technology (Lister *et al.*, 2008, Mortazavi *et al.*, 2008, Nagalakshmi *et al.*, 2008). Unlike microarrays, RNA-Seq does not depend upon a fully annotated genome for probe selection and so there are no related biases and a whole-genome transcriptome map can be created by aligning the reads to a reference genome, which enables detection of splice variants. This is important as 92-94% human genes undergo alternative splicing, with many of the variants specific to tissues and individuals (Wang *et al.*, 2008). RNA-Seq does however pose challenges for data analysis and storage (Mutz *et al.*, 2013).

The generation of mature mRNA in the nucleus requires pre-mRNA processing. This includes a 5' cap addition, splicing and polyadenylation (polyA tail) to the 3'end of the majority of eukaryotic mRNAs. The polyA tail provides stability and has important roles in nucleocytoplasmic export and translation (Moore & Proudfoot, 2009). RNA can be categorised as polyadenylated (polyA+/ polyA) or non-polyadenylated (non-polyA/ polyA) transcripts, depending upon the addition of the polyA tail at the 3' end. mRNA-Seq involves the enrichment of polyA RNAs by oligo(dT) selection. mRNA-Seq, like total ribodeleted RNA-Seq also provides splicing information (Pan *et al.*, 2008).

The selection for polyA transcripts excludes the detection of transcripts that are not polyadenylated, including functional long transcripts (>200 bp) such as long non-coding RNAs (lncRNAs) (Wilusz *et al.*, 2008), ribosomal RNAs (rRNAs) and replication dependent histone mRNAs (Marzluff *et al.*, 2008, Yang *et al.*, 2011). There are also transcripts which

are bimorphic, which cannot be categorised as either polyA+ or polyA- owing to processing which either reduces or removes the polyA tail, and examples of excised introns that have been identified as a new class of stable non-polyadenylated lncRNAs (Yang *et al.*, 2011). Several lncRNAs have been attributed functions in immunity (Geng & Tan, 2016) and biological processes such as alternative splicing (Yin *et al.*, 2012), transcriptional regulation (Zhang *et al.*, 2013), and microRNA regulation (Hansen *et al.*, 2013, Memczak *et al.*, 2013). The functions may include acting as decoys, scaffolds or enhancer RNAs that contribute to chromatin rearrangement (Fang & Fullwood, 2016). Despite recent advances, most lncRNA functions remain to be determined (http://fantom.gsc.riken.jp/), owing to the multiple mechanisms and complex dynamics involved in lncRNA expression involving chromatin state, core transcription factors and the post transcriptional regulation involving microRNAs (Wu *et al.*, 2014). Many non-polyadenylated lncRNAs are lowly expressed yet highly tissue- and species- specific, with poor evolutionary conservation. The processing of non-polyadenylated lncRNAs is closely linked to splicing of the gene from which they were derived (Zhang *et al.*, 2014). lncRNAs which are conserved across species can provide important information regarding their functional roles (Li & Yang, 2017, Bush *et al.*, 2018). Therefore, to improve understanding of the regulatory network governing expression of the sheep genome, this project analyses both the polyA and non-polyadenylated transcriptional landscape of the sheep.

RNA-Seq does have certain limitations in assembling lncRNA transcripts (Kukurba & Montgomery, 2015), as the assembled transcriptome includes partial transcripts and artefacts due to fragment bias (due to non-uniformity of cDNA fragments within the transcripts they represent) (Roberts *et al.*, 2011). The short sequence fragments generated in RNA-Seq (Trapnell *et al.*, 2009) mean that for those genes that are expressed at a very low level, there may be gaps in their assembly which can lead to mistaken annotation as lncRNAs (Kozarewa *et al.*, 2009, Sun *et al.*, 2012).

This project is utilising both polyA-selected library preparation protocols and ribodepleted protocols to enable the capture of all types of transcripts. The reads are 125bp in length which provides a balance between accuracy in sequencing, splicing detection and cost (Chhangawala *et al.*, 2015). All reads were paired-end which enables both ends of the fragment to be aligned, improving the quality of detection of genomic rearrangements, repetitive sequence elements, splice variants and novel transcripts.

As described in Chapter 2 and shown in Figure 2.2, two pipelines were used to map transcripts and assign transcript and gene expression values. Kallisto2 (Kallisto) rapidly and accurately pseudo-aligns reads to a reference transcriptome, providing accurate expression estimates for each reference transcript in OarV3.1. The StringTie pipeline utilises more computing power, time and two methods of precise read alignment (HISAT2 and BOWTIE2) to map reads to the reference genome, then StringTie assembles these alignments enabling gene expression estimates to be determined, as well as revealing novel transcript models. With novelty comes potential errors in those transcripts which are very lowly expressed, so both pipelines have been utilised to maximise accurate analysis of the reference transcriptome as well as provide information on novel transcript expression. Every sample submitted was processed using both pipelines and the resulting expression estimates are examined in Chapters 4 and 5. In addition, two library preparation methods were used to capture either polyadenylated RNA (25M read depth), or total RNA depleted for rRNA (100M read depth) for both LPS stimulated and unstimulated BMDM samples from each individual.

This chapter compares the two preparation methods and the two analysis pipelines and finishes with a very preliminary examination of the repeat regions (reads not utilised by Kallisto) which contribute to the 'dark matter' of genomic sequence that currently has unknown function within the sheep genome. How much of this 'dark matter' is junk or has unknown function is under scientific debate. The evolutionary significance for 'dark matter' is that it partly accounts for the deleterious mutational load in a genome, with the functional fraction within the human genome unable to exceed 25% (Graur, 2017). The Encyclopedia of DNA Elements Project (ENCODE) (Consortium, 2012) has revealed that biochemically active regions cover a much larger fraction of the genome than evolutionarily conserved regions, with chromatin profiling (Ernst *et al.*, 2011, Gerstein *et al.*, 2012, Trynka *et al.*, 2013) and many GWAS studies showing human disease susceptibility loci to lie outside protein coding regions (Kleinjan & van Heyningen, 2005, Maurano *et al.*, 2012, Kellis *et al.*, 2014).

3.2 Comparison of the outputs of Kallisto2 and StringTie Pipelines

Kallisto is a fast, efficient and accurate program (Bray *et al.*, 2016), used for quantifying abundances of transcripts from RNA-Seq data of known target sequences from a reference transcriptome (see Chapter 2.8.1). The expression level estimate for each reference

transcript from OarV3.1.81 is provided as transcripts per million (TPM), a measurement of the proportion of transcripts in the RNA sample pool.

For the purpose of improved annotation of the reference sheep genome, to identify novel transcripts, splice variants and intron/exon boundary information, a second pipeline StringTie (Pertea *et al.*, 2016) utilised a two pass approach (see Chapter 2.8.2) to alignment, making use of HISAT2 as the priority aligner, with the relatively stringent, rapid and accurate alignment to the reference (mapping 73-86% of raw reads, see Table 3.1). The unmapped reads from HISAT2 were passed to BOWTIE2, another aligner which has a local alignment tool to 'soft clip' the ends of the read that maybe inaccurate. Based upon this tool, previously unmapped HISAT2 reads were 'rescued' and utilised in the analysis. With the combined approach, >99% of the reads were used to inform further analysis, as demonstrated in Table 3.1. Mapping is primed from the 5' end so any adaptor contamination at the 3' end can be trimmed by the alignment step. StringTie creates a list of all gene transcripts identified and the expression level estimates are provided as Fragments per kilobase of exon per million reads mapped (FPKM). There are multiple aligners available and it is acknowledged that this is a fast-developing field.

Table 3.1 is a simplified version of Appendix 3.1 and shows that 55-68% of all raw reads were used in estimating transcript abundance (including coding and non-coding reference transcripts) using Kallisto, compared to over 99% of all raw reads being mapped and utilised by the StringTie pipeline. The remaining 32-45% of reads that were not utilised by Kallisto are further explored and explained in Section 3.3.

Table 3.1 Comparative analysis of alignment rates for different analysis pipelines
This table compares pseudo-alignment rates from Kallisto2 (Kallisto) with the average StringTie alignment rates after merging HISAT2 and Bowtie2 for each sample

	AVG % Pseudoaligned with Kallisto2	**AVG % Aligned with HISAT2 alone**	**AVG % Aligned after HISAT2 and BOWTIE2**
0hr- PolyA /mRNA	67.4	84.4	99.3
2hr - PolyA/mRNA	55.7	78.9	99.1
4hr- PolyA/mRNA	64.7	79.0	99.4
7hr- PolyA/mRNA	66.2	85.7	99.4
24hr- PolyA/mRNA	65.6	80.6	99.4
0hr – ribodepleted/ TOTAL RNA	60.8	75.4	99.4
7hr- ribodepleted/ TOTAL RNA	59.1	73.6	99.2
PBMC-PolyA/mRNA	54.9	76.6	99.2
AM- PolyA/mRNA	66.1	80.0	99.4
MDM- PolyA/mRNA	65.7	77.9	99.2
BL- PolyA/mRNA	57.0	86.3	99.5

From the earliest gene discovery efforts by the FANTOM consortium, mouse macrophages have been shown to have a very complex transcriptome with many novel mRNAs (Wells *et al.*, 2003, Baillie *et al.*, 2017). Analysis of the sheep macrophage transcriptome revealed the same complexity. The reference transcriptome in the sheep is primarily focussed on the protein-coding transcriptome, and the vast majority of genes are represented by a single transcript. The Kallisto pipeline based upon polyA+ mRNA identified 23,133 transcripts from amongst 28,757 reference transcripts (80.4%) having expression estimates > 0 TPM in any individual in the sheep macrophage transcriptome. Among the total RNA

libraries (0hr and 7hr) with the greater sequencing depth, 23828 transcripts (82.6%) were found to have expression estimates > 0 TPM in at least one individual.

Given the diversity of the protein-coding transcriptome, there was an expectation that macrophages would also be a rich source of non-coding RNAs. One class of lncRNAs is derived from the transcription of enhancers. The stimulation of mouse macrophages with LPS was associated with transcription of more than 3,000 enhancer RNAs (Kaikkonen *et al.*, 2013). Some, but not all, of these transcripts are likely to be polyadenylated but most are unspliced. StringTie is a discovery pipeline and discovery/annotation was the rationale for sequencing the total RNA libraries using the key samples (resting 0hr and maximal response 7hr LPS samples) at a greater depth of 100 million reads/sample. This method does not positively select for polyadenylation so captures non-polyadenylated protein coding and noncoding transcripts. The greater depth of sequencing detects the rarer and lower expressed transcripts with more coverage and confidence from a wide range of RNAs.

The total number of transcript models identified by StringTie for the PolyA immune cell samples alone was 91,535, more than three times the number of transcripts currently recognised in the reference transcriptome OarV3.1.81 (Li *et al.*, 2015) that Kallisto utilised, primarily due to the multiple splice variants that StringTie is able to detect. Fig 3.1 summarises the outcomes of the two independent pipelines. Each pipeline produces a final expression level estimate (TPM for Kallisto and FPKM for StringTie), for every transcript within the overall index; Kallisto utilising purely the reference transcripts from OarV3.1.81; whereas StringTie created an index of non-redundant transcript models across all samples and time points, merging all models created using HISAT2 and BOWTIE2, as well as the reference transcriptome from OarV3.1.81. Examining the maximum transcript expression across all individuals and BMDM response samples, for every transcript within each respective index enables a comparison of the two pipelines and it is possible to visualise how many more transcript models are robustly detected through using the StringTie pipeline.

Mammalian transcriptomes display a power-law distribution in transcript abundance; in simple terms there are few very highly expressed and many lowly expressed genes and a log transformation produces a straight line (Ueda *et al.*, 2004). Figure 3.1 shows the distribution of expression estimates for the two pipelines separated into classes of abundance. The analysis shows that at expression level cut offs (FPKM >5 for StringTie and

TPM >1 for Kallisto), the power law relationship fails. Below this level of expression, there is greater noise because the level of coverage from RNA-Seq is insufficient to overcome the stochastic sampling effects.

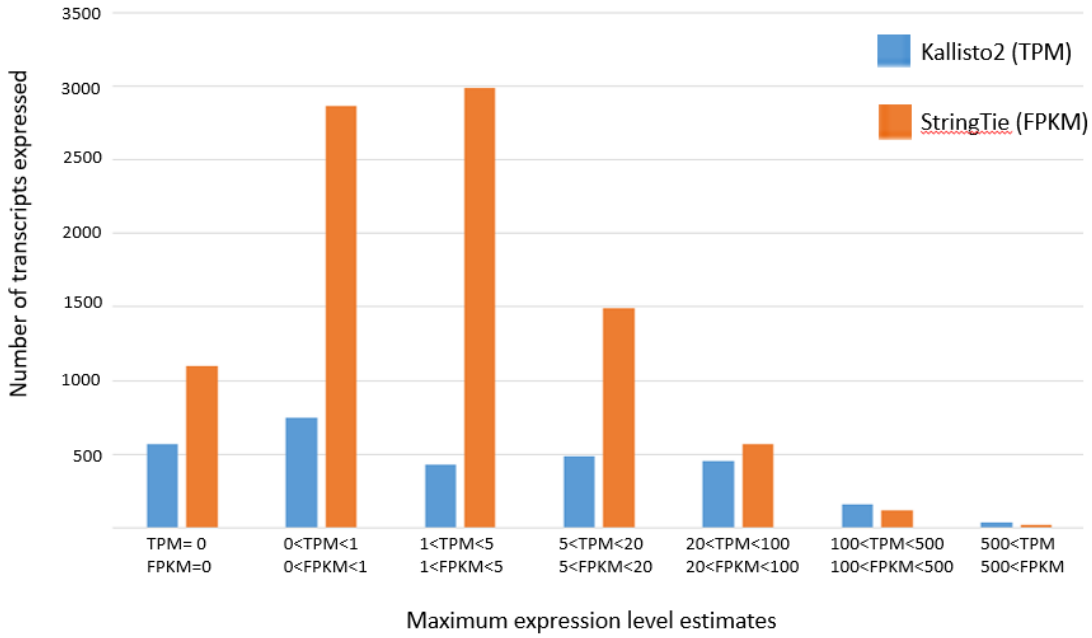


Figure 3.1 The distribution of maximum transcript expression estimates for StringTie (FPKM) and Kallisto (TPM) pipelines
The numbers of transcripts with different maximum expression levels across all time points in the polyA-selected time course (0hr, 2hr, 4hr, 7hr and 24hr) over all individuals were compared to highlight the differences between the results from Kallisto and StringTie. The X-axis shows the different expression level (TPM/FPKM) class boundaries; (0,0-1, 1-5, 5-20, 20-100, 100-500, > 500). The Y axis shows the number of transcripts expressing a maximum expression estimate (the maximum expression detected in any individual or sample) within that expression boundary.

For every merged HISAT2 and Bowtie2 BAM file, SAMtools flagstat was used to provide some simple statistics including information on how many reads were included in each file, the pairing of these reads and how these pairs were mapped (see Appendix 3.2). 77-90% (Average 85%) of all paired reads were properly paired across all the samples.

To cross validate these two pipelines which estimate gene expression, the sum of mean transcript expression estimates for each reference gene using Kallisto (TPM) was compared to the mean reference gene expression estimates obtained using StringTie version 1.3.3 (FPKM), using the GffCompare utility which is part of the Cufflinks/Tuxedo suite (Pertea *et*

al., 2015). The expression estimates from StringTie for each reference transcript, had to merge the expression assigned to novel transcript models that were found for many of the reference OarV3.1 genes (such as *CSF1R,* see Ch.2.8.2). All expression estimates had 0.00000001 added prior to the Log10 calculation, to avoid any divisions of 0, and both the Pearson's product-moment correlation (r) and Spearman's rank-order correlation (Rho) were calculated, removing all expression estimates of 0 prior to calculating the Log 10 correlations r and Rho.

As noted above in Figure 3.1, and below in the scatterplots, consistent with the known power law relationships of transcript abundance observed in many other systems (Ueda *et al.*, 2004, Lu & King, 2009), a relatively small number of transcripts contributed the majority of reads in a sample. There was minimal differential expression detected in some of the most abundant protein coding transcripts which appear as outliers in both basal (0hr LPS) and peak (7hr LPS) activation expression profiles. These outliers include *SPP1, GSTCD* and protein coding genes lacking any functional annotation such as *ENSOARG00000014339* (subsequently annotated as *Ferritin heavy chain FTH1*).

Figures 3.2 and 3.3 compare the different pipeline expression estimates for 0hr and 7hr LPS treated BMDMs respectively. All plots show a positive correlation between the expression estimates derived from StringTie (FPKM) and Kallisto2 (TPM), with most reference genes from OarV3.1 demonstrating similar expression profiles, irrespective of the pipeline used to obtain them.

It is interesting to note that despite the similar broad appearance of the correlation profiles between the plots, Pearson's product-moment correlation (r) differs in value quite dramatically between the different plots, and is more stringent when compared to Rho. This is because of the numbers of transcripts depicted in each plot, the majority of which are not visible and Pearson correlation evaluating the linear relationship between the two pipeline expression estimates, treating the estimates as continuous, which is in contrast to the Spearman correlation which is based on ranked expression values for each pipeline rather than the transcript expression levels themselves.

The disparity in completeness of the reference transcriptome between the protein coding transcriptome (selected by PolyA libraries) and the total ribodepleted transcriptome has also clearly had an effect when trying to compare the two pipelines.

Figure 3.2 Correlation of Kallisto and StringTie gene expression estimates of resting BMDMs (at 0 hr LPS)
Using A: PolyA selected libraries and B: Ribodepleted total libraries, i) and ii) show correlation plots between Kallisto (tpm) and StringTie (FPKM) for i) the average (mean) and ii) Log10 (mean + 0.00000001) expression of Ensembl ID genes across all six individuals for 0hr LPS samples for both library preparation methods. The genes are coloured according to gene type (assigned using Biomart) according to the key C.
The Pearson's product-moment correlation is provided as r, and Spearman's rank-order correlation is provided as Rho, to provide numerical strength of the association and link respectively (for Log10 plots, all expression estimates of 0 were removed).

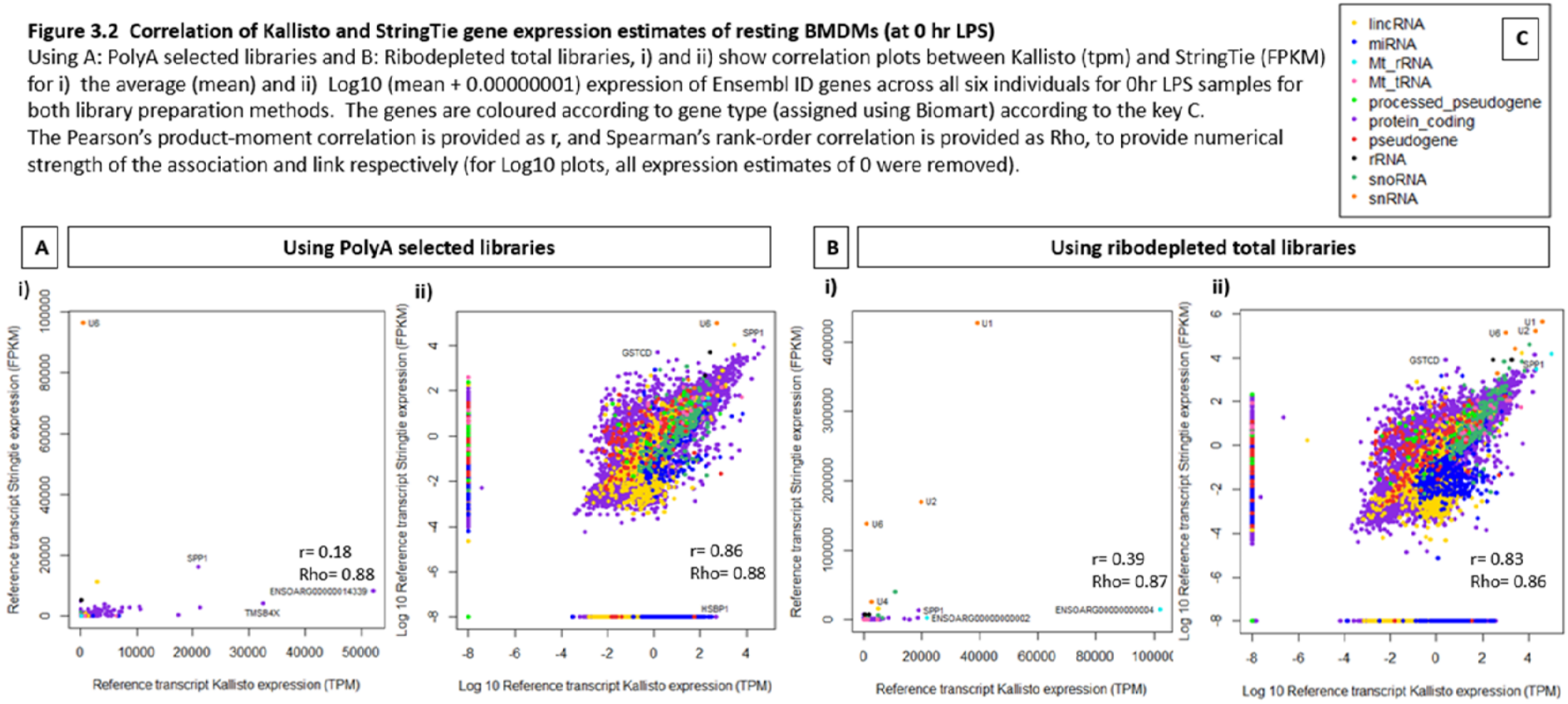
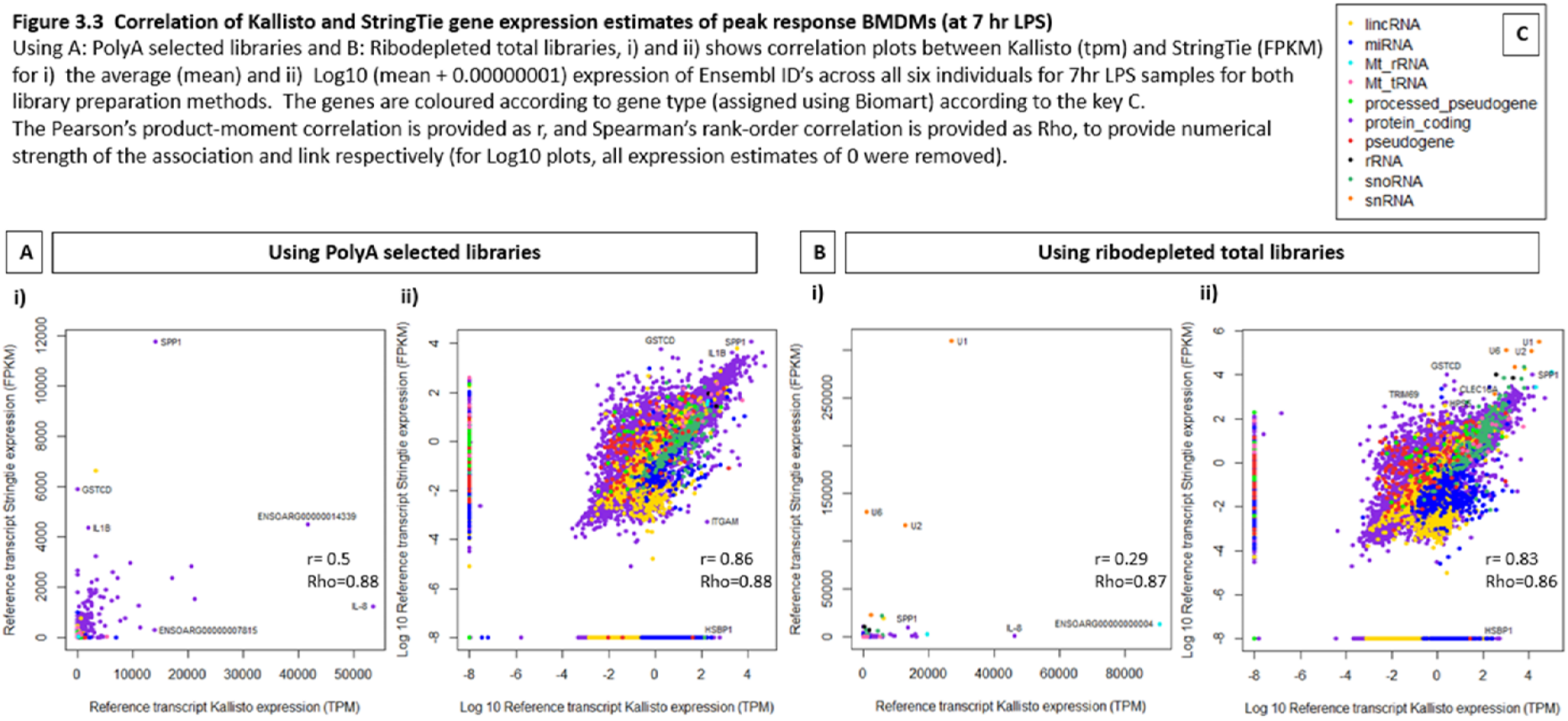


Figure 3.3 Correlation of Kallisto and StringTie gene expression estimates of peak response BMDMs (at 7 hr LPS)
Using A: PolyA selected libraries and B: Ribodepleted total libraries, i) and ii) shows correlation plots between Kallisto (tpm) and StringTie (FPKM) for i) the average (mean) and ii) Log10 (mean + 0.00000001) expression of Ensembl ID's across all six individuals for 7hr LPS samples for both library preparation methods. The genes are coloured according to gene type (assigned using Biomart) according to the key C.
The Pearson's product-moment correlation is provided as r, and Spearman's rank-order correlation is provided as Rho, to provide numerical strength of the association and link respectively (for Log10 plots, all expression estimates of 0 were removed).



In the $\log_{10}$ transformed plots of Figures 3.2 and 3.3, irrespective of library type or activation state StringTie was generally better able to assign expression of protein-coding genes and pseudogenes (represented by purple and red dots producing a 'hump' to the left of line of best fit), whilst Kallisto appears to assign greater expression estimates for lincRNAs and miRNAs (represented by navy blue and yellow spots producing a 'hump' to the right of line of best fit). Differences in the majority of protein-coding genes are likely to be attributable to StringTie's ability to detect and assign expression to multiple novel transcript models for those given genes, as Kallisto is constrained to assigning expression only to the reference transcript models, which may not be the most highly expressed model, as demonstrated by *Interleukin 1 beta (IL1B)* (see Chapter 5.4.7). With lincRNAs and miRNAs generally representing shorter length transcripts, the accuracy of Kallisto in identifying these small transcript lengths explains why Kallisto is better able to assign expression estimates as opposed to StringTie, which associates expression of these transcripts with other transcript models.

Both pipelines identified the *C-X-C motif chemokine ligand 8 (CXCL8),* highlighted in Figure 3.3.A.i and B.i as *IL-8*, to be highly expressed in the peak activation samples. IL-8 is a granulocyte chemoattractant and is not present in rodent genomes but is one of the most highly LPS-induced transcripts in human, pig and horse macrophages (Nieto *et al.*, 2009, Kapetanovic *et al.*, 2012, Baillie *et al.*, 2017). StringTie identified 16 different transcript models. The most similar reference transcript model was not the most highly-expressed model (*MSTRG.27919.16*). Kallisto oversimplifies the impression of gene level expression since most genes have several transcript models. Nevertheless, the pseudoalignment has actually provided a relatively accurate level of gene expression, for which functional annotation is generally sufficient. StringTie has provided gene level expression estimates and highlighted possible alternative splice isoforms. These novel transcript models can therefore improve upon the current reference OarV3.1.

There are significant differences between the pipelines. StringTie has assigned high expression to Glutathione S-transferase C-terminal domain containing (*GSTCD*) in all samples, assigning a mean expression of 4962 FPKM, whereas Kallisto assigned a mean expression of only 1.6 TPM across all samples. On closer inspection this is because Gff Cuffmerge has merged both *MSTRG.27343*, which has the same transcript model as *ENSOART00000010840 (GSTCD)* containing 11 exons, and *MSTRG.27345* which is a novel

transcript model containing only one exon. Therefore, in some cases Kallisto is providing the more accurate expression of reference gene expression. Neither humans, mice or pig macrophages express *GSTCD* to meaningful levels (Schroder *et al.*, 2012, Kapetanovic *et al.*, 2013, Baillie *et al.*, 2017). It is therefore worth curating the specific cases where the two pipelines differ.

In other protein-coding genes, Kallisto has assigned significant expression where StringTie does not. One example includes *integrin subunit alpha M* (*ITGAM*), highlighted in Fig 3.3.A.ii, which was assigned a mean expression of 204 TPM by Kallisto whereas StringTie only assigned a mean of 4 FPKM. This gene encodes for the CD11b integrin which is known to combine with ITGB2 to form macrophage receptor 1 (Mac-1), important in macrophage adhesive interactions (Fagerholm *et al.*, 2006), and therefore robust transcript expression was expected in sheep BMDMs. *ITGAM* only has one, complex transcript model in OarV3.1, with 31 exons, unlike the 1:1 orthologue with humans (with highly conserved regions) where 8 transcript variants have been identified, varying in length and numbers of exons (Aken *et al.*, 2016). *ITGAM* is situated within a particularly complex region of chromosome 24, with many genes with equally complex transcript models such as *ITGAX* (identified as having 16 transcript models with up to 58 exons, assigned a mean expression of 160 FPKM), and *ITGAD* (3 transcript models with 31 or 14 exons, assigned an expression of 11 FPKM). The StringTie algorithm was not efficient in accurately discriminating between these transcript and gene models.

ENSOARG00000019602/ ENSOART00000021345, annotated as *heat shock factor-binding protein 1 (HSBP1)* is highlighted in Fig 3.2.A.ii, and represents another example of disparate assignment of expression between the two pipelines. Kallisto assigned a mean expression of 524 TPM across all PolyA-selected LPS response samples, where StringTie did not detect any expression of *HSBP1.* On closer examination of the region in IGV, there is no coverage over the specified location (Chr 2: 125,821,048-125,821,259). OarV3.1 shows only one transcript model, which is 204 base pairs in length, containing only one exon, however there is virtually no synteny when the region is compared with mice or humans using Ensembl. Both mice and humans have multiple transcript variants for *HSBP1* with 2,3 or 4 exons (Li *et al.*, 2015). This highlights the need for two independent pipelines to examine gene expression in these samples, as the reference transcriptome is not always correct. It is

likely that the new assembly of sheep, and related high resolution goat assembly will provide greater clarity.

As expected, the greatest proportion of 'novel' transcript models came from the ribodepleted total RNA samples which were sequenced at the greatest depth. The novel models detected at very low expression levels, with low coverage, are less likely to be reproducible, which explains the apparent divergence between expression estimates derived from each pipeline in the lowly expressed genes.

3.3 Identification of differentially-expressed transcripts

Due to the biological variation that exists between individuals, the random sampling intrinsic to RNA-Seq, possible technical variation between RNA-Seq sequencing library preps(Oshlack *et al.*, 2010), and the multiple sampling that is intrinsic to genome-scale analysis, determining what constitutes a "significant" change in transcript abundance is not straight-forward.

The perfect RNA-Seq experiment would identify the counts of reads to be proportional to the abundance of transcripts that they originate from and follow an approximate Poisson distribution with an error rate that decreases as the depth of sequencing is increased (Bullard *et al.*, 2010). Pseudogenes and gene families have very similar sequences which creates a problem for Kallisto as allocating 'counts of reads' to a specific transcript or gene can be unreliable. If genomic features have been considered, as with StringTie, reads are no longer ambiguous, technical replicates are unnecessary and the focus is entirely on modelling the biological variation between samples (Trapnell *et al.*, 2010). The process of estimating transcript level counts with methods that do utilise precise mapping can still introduce some technical variation (Pimentel *et al.*, 2017).

Sleuth is a program that was created to bridge the gap between count-based methods and quantification algorithms by fully exploiting the advantages of both (Pimentel *et al.*, 2017). Sleuth utilises the bootstraps of Kallisto2 which serve as proxies for teasing apart the technical variance from the biological variance, enabling a "true" estimate of the biological variance. In simulated data Sleuth appeared to outperform other 'count based' statistical packages such as DEseq2, edgeR and voom (Pimentel *et al.*, 2017).

Ballgown is a program able to take output files from the StringTie pipeline and implement a basic linear modelling strategy to rigorously apply statistical tests to determine differential

expression at the exon, gene or transcript level. FPKM values are skewed so the variance is stabilized by the programme applying a log transformation and then fitting a standard linear model. At the time of writing this thesis there was no better statistical software that allows the same level of flexibility for modelling transcript-level data (Frazee *et al.*, 2015). Transcripts expressed at lower levels are more difficult to assemble and spurious models can produce misleading apparently significant differences (Bush *et al.*, 2018). There has not been any pre-filtering of transcripts prior to running Ballgown or Sleuth, so the p and q values must be qualified by looking at the estimated expression levels of that given transcript or gene model. Segregation of counts amongst different transcript models reduces the statistical power; so it is intrinsically easier to demonstrate differential expression at the gene, rather than the transcript level (Pertea *et al.*, 2016).

Both Sleuth and Ballgown provide a p value and q value indicating the accuracy of the estimates of differential expression, and can account for confounding variables, which for the purpose of this project included sex when focusing on LPS time point. The null hypothesis is that transcripts do not change in expression between time points. The p value is the probability of finding a given transcript to be differentially expressed when it is in fact not, it takes into account the mean expression and sample size and relies upon establishing the size of the difference in expression in comparison to the variance to estimate significance. A biologically meaningful value for this significance cut off is still the subject of debate (Fatovich & Phillips, 2017). With a p value of 5%, multiple tests on the same sample as with RNA-Seq can result in a large number of false positives, therefore the p value needs to be adjusted. The q value takes into account the false discovery rate (FDR), and Ballgown and Sleuth both utilise a false discovery rate (FDR) to account for multiple testing (q value). The q value controls the estimated proportion of false positives among the significant results. It is therefore more stringent than the uncorrected p value but directly correlated with it. When ranking the significance of a given set of differentially expressed transcripts, the order remains the same regardless of whether p or q values are used.

The two approaches to identify significant differences are difficult to compare as Sleuth examines Kallisto results at the transcript level of expression, whilst Ballgown examines StringTie gene level expression. Miru does not assign a p or q value to an individual node (transcript or gene), or account for confounding variables, however the clustering is entirely based on valid statistical methods, algorithms and matrices (discussed in 2.9) and provides

a more useful visual approach to extracting biologically meaningful transcripts and genes. For completeness, the respective p and q values derived using either Sleuth for the Kallisto transcript expression networks or Ballgown for the StringTie gene expression networks have been assigned to the final 'peaking analyses' workbooks in Chapter 5.

3.4 Ribodepleted Total RNA compared to PolyA-selected mRNA libraries

For the LPS time course, there were two methods of library preparation utilised by Edinburgh Genomics (see chapter 2.7.1). For all individuals, all five LPS timepoints were used to generate PolyA-selected libraries and paired end reads at 25M read depth were used to analyse mRNA expression. The 0hr and 7hr samples from each individual were also subjected to the alternative ribodepleted (total) RNA protocol and sequenced with paired end reads at 100M read depth. The same approach was used to analyse a subset of libraries in the wider sheep transcriptional atlas (Clark *et al.*, 2017)

Total RNA libraries removed the abundant rRNAs through hybridization capture of rRNA followed by binding to magnetic beads. The better the efficiency in removing rRNA, the greater the numbers of reads that will map to transcripts of interest, as rRNAs make up >80-90% of the total RNA of an un-enriched sample(O'Neil *et al.*, 2013). As shown in a detailed comparative analysis of HEK293 cells, the ribominus protocol enriches for detection of non-poly-adenylated transcripts, but also captures introns from unprocessed nuclear transcripts (Sultan *et al.*, 2014). These can potentially be merged for expression estimates, but there is an additional benefit for the future study of allele-specific expression, and specific regulatory variants (single nucleotide variants; SNVs), since intronic transcripts are greatly enriched for SNVs (Sale *et al.*, 2007, Kumar *et al.*, 2017) and it is known that a SNV may profoundly alter transcription factor (TF) binding sites and significantly contribute to regulatory variation within the genome (Kumar *et al.*, 2017).

The differential detection of transcripts in the two library methods clearly has an impact on gene expression estimates. Figure 3.4 shows a principal component analysis (PCA) plot for all of the libraries from the LPS time course. The scale of the response to LPS is evident from the clear impact of time as a variable (detected by PC2), but library type also separates samples from the same time points (0 and 7 hours) (PC1).

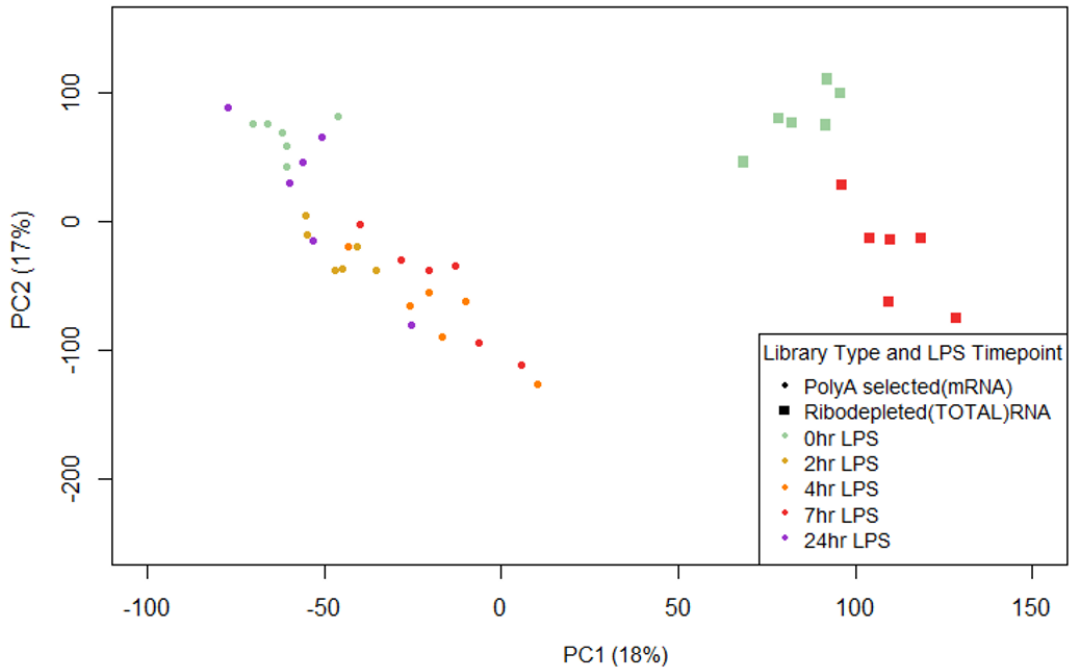


Figure 3.4 PCA to demonstrate library type differences
PCA plot using transcript expression data derived from Kallisto2 for all LPS samples from both polyA-selected libraries and ribodepleted total libraries, depicted as circles and squares respectively. The points are coloured according to LPS time point with 0hr being turquoise, 2 hr yellow, 4 hr orange, 7hr red and 24 hr purple. The percentage of variance is shown in brackets for each principal component.

In Chapters 4 and 5, the results from the two library types were separated for analysis and utilised independently of one another. The PolyA-selected mRNA time course was used to examine the global transcriptional changes that take place when sheep BMDM respond to LPS with the focus on polyadenylated mRNAs which will be destined for translation. The ribodepleted total RNA time points from 0hr and 7hr at the greater sequencing depth remain to be explored more thoroughly. Due to the increased costs involved in sequencing ribodepleted total RNA samples at the greater sequencing depth, it was not possible to do this sequencing for all the time points.

3.5 Differential detection of transcripts with different library preparation

Figures 3.5 and 3.6 show scatterplots comparing the expression detected by the polyA plus and ribodepleted libraries, analysed using both the Kallisto (TPM) and StringTie (FPKM) pipelines. As demonstrated in Figures 3.5 and 3.6, despite the difference in sequencing depth, the vast majority of genes demonstrate a positive correlation in expression estimate, irrespective of library method utilised. However, neither selection protocol was 100% efficient at removing undesired sequences and retaining desired sequences. Known abundant non-poly-adenylated transcripts were detectable in the polyA-selected libraries despite the lower depth of sequencing.

Figure 3.5 Scatter plots to demonstrate correlation of gene expression estimates at 0hr LPS, detected by different library types
A: Using StringTie and B: Using Kallisto, i) and ii) shows the correlation plots between polyA selected and ribodepleted total library types for i) the average (mean) and ii) Log10 (mean + 0.00000001) expression of Ensembl ID's across all six individuals for 0hr LPS samples for both pipelines. The genes are coloured according to gene type (assigned using Biomart) according to the key C. The Pearson's product-moment correlation is provided as r, and Spearman's rank-order correlation is provided as Rho, to provide numerical strength of the association and link respectively (for Log10 plots, all expression estimates of 0 were removed).

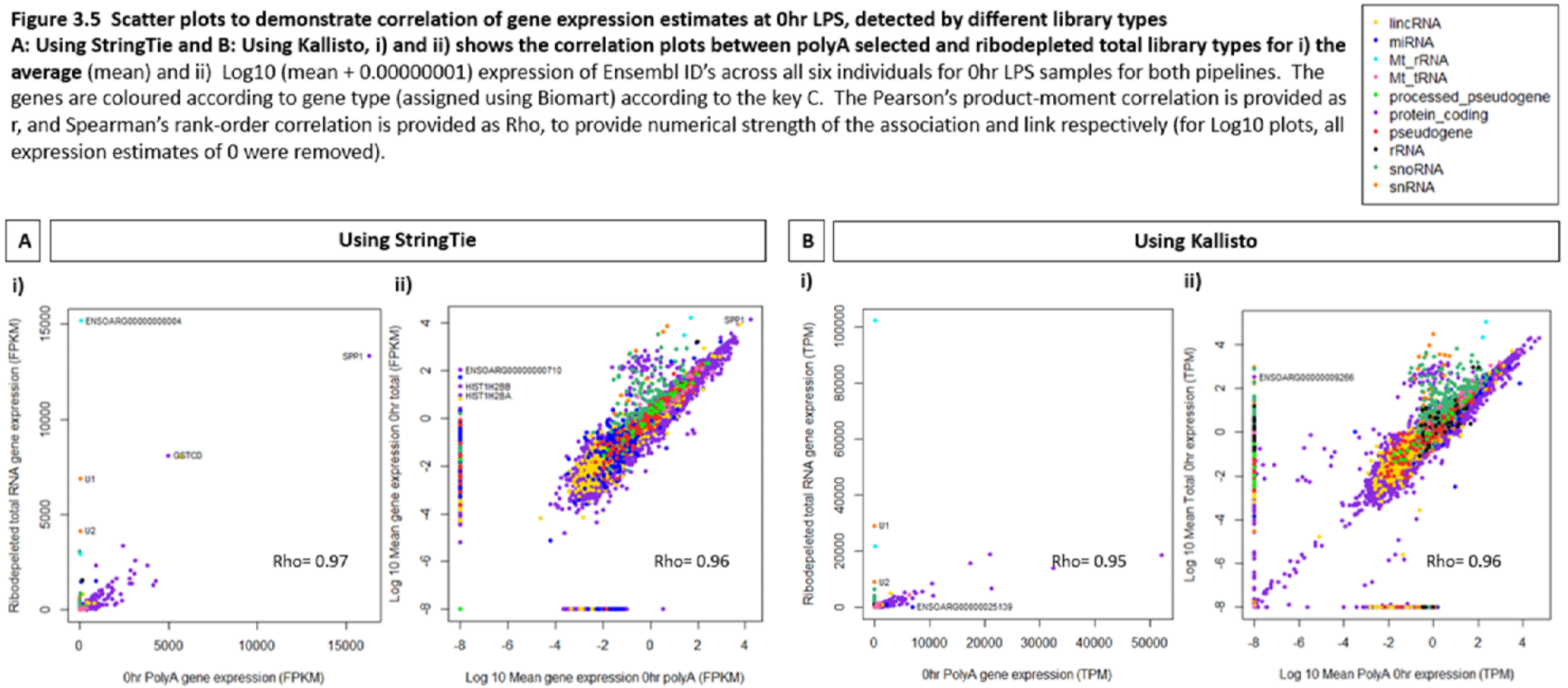


Figure 3.6 Scatter plots to demonstrate correlation of gene expression estimates at 7hr LPS, detected by different library types
A: Using StringTie and B: Using Kallisto, i) and ii) shows the correlation plots between polyA selected and ribodepleted total library types for i) **the average** (mean) and ii) Log10 (mean + 0.00000001) expression of Ensembl ID's across all six individuals for 7hr LPS samples for both pipelines. The genes are coloured according to gene type (assigned using Biomart) according to the key C. The Pearson's product-moment correlation is provided as r, and Spearman's rank-order correlation is provided as Rho, to provide numerical strength of the association and link respectively (for Log10 plots, all expression estimates of 0 were removed).

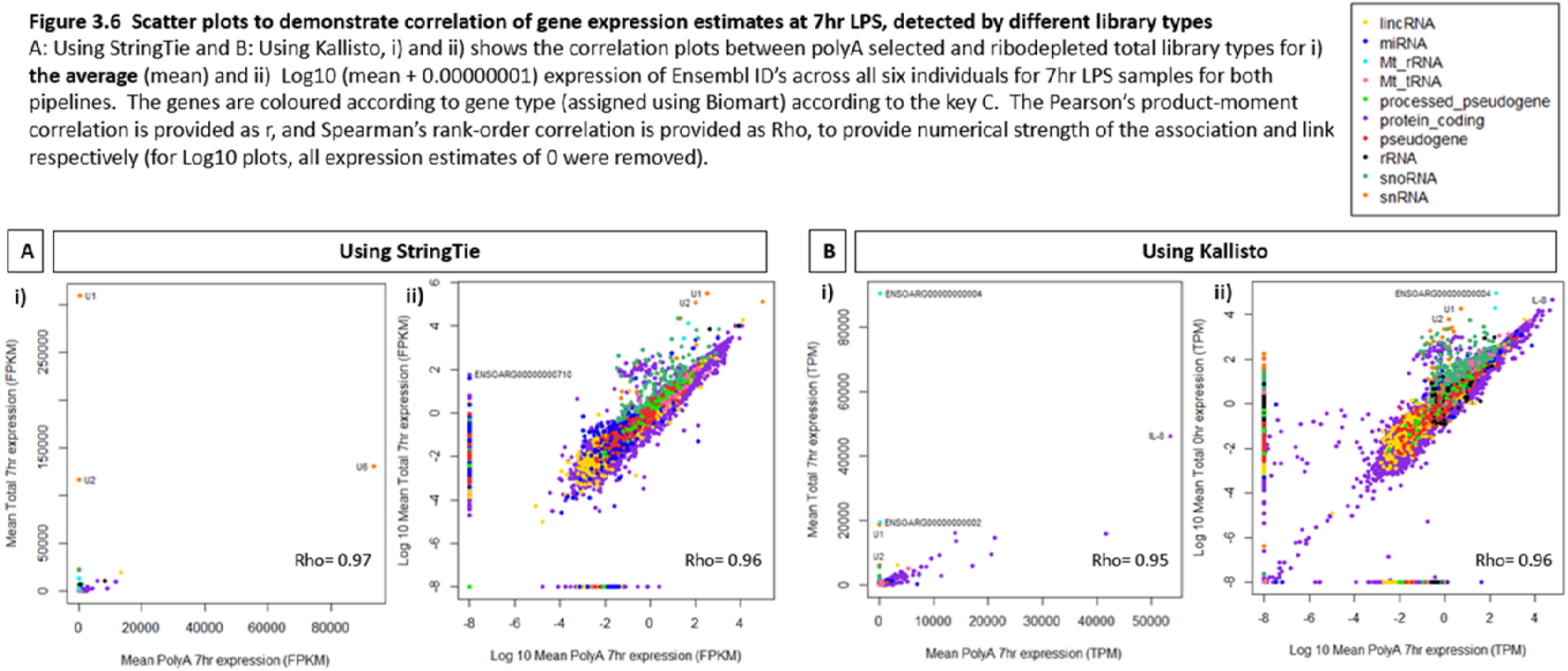


Figure 3.7 utilises data derived from Kallisto and shows a 92% overlap in the reference transcripts (OarV3.1.81) that were detected with expression > 0 TPM that are detected for each of the library types. Table 3.2 expands upon this information to show the breakdown of detection of different types of reference transcript in each library type, with the greatest difference to be in the detection of protein-coding transcripts for the polyA-selected libraries, and for the ribodepleted total libraries in the detection of miRNA, snoRNA , snRNA and to a lesser extent rRNA.

The majority of transcripts detected that were selectively enriched by the ribo-minus protocol, were either snoRNAs or snRNAs. Most of the protein-coding genes/transcripts within this group required functional annotation and were found to be histone mRNAs which are probably not polyadenylated (Dávila López & Samuelsson, 2008, Marzluff *et al.*, 2008). All other protein coding genes over-represented in in the ribodepleted total libraries, with reliable levels of detection (some of which had associated annotation), were not significantly LPS responsive or macrophage specific and therefore not relevant to the analysis of transcriptomic changes associated with macrophage biology, the subject of this project.

Figure 3.7 Venn diagram of all OarV3.1 transcripts detected using Kallisto, for each of the library types used for 0hr and 7hr LPS samples.
Transcripts with a Kallisto expression estimate (TPM >0), detected in any BMDM sample (either 0hr or 7hr) in the polyA-selected and ribodepleted total library types. The percentages indicate the proportion of transcripts within the reference transcriptome OarV3.1.81

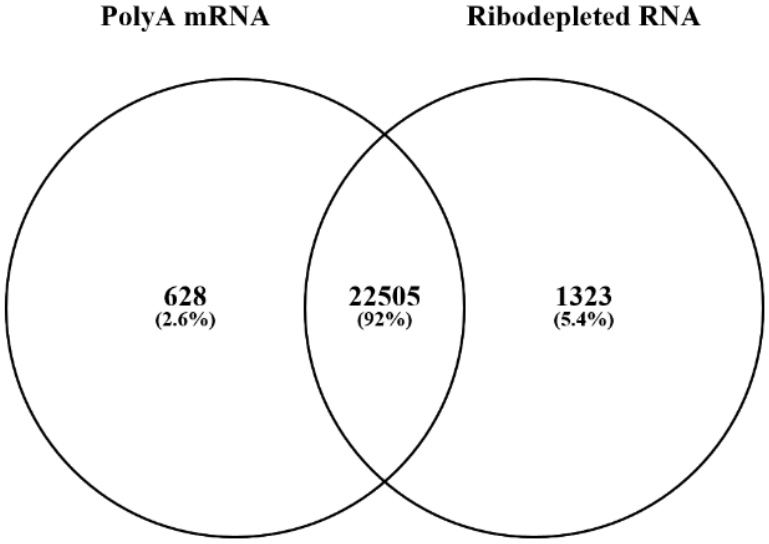


Table 3.2 Numbers and proportions (%) of the different types of transcript detected in each of the library types.
The table shows the numbers and proportions of different types of transcript that were detected in each of the library types (polyA-selected or ribodepleted total), which showed a Kallisto expression > 0 TPM in any individual for either 0hr or 7hr LPS sample.

Transcript Type	In PolyA, not total	In total, not PolyA	In polyA, not total (%)	In total, not polyA (%)
lincRNA	33	58	5.3%	4.4%
miRNA	26	196	4.1%	14.8%
processed_pseudogene	1	0	0.2%	0.0%
protein_coding	506	754	80.6%	57.0%
Pseudogene	8	22	1.3%	1.7%
rRNA	12	44	1.9%	3.3%
snoRNA	15	70	2.4%	5.3%
snRNA	27	179	4.3%	13.5%
TOTAL	**628**	**1323**		

3.6 Examination of repeat regions within genome

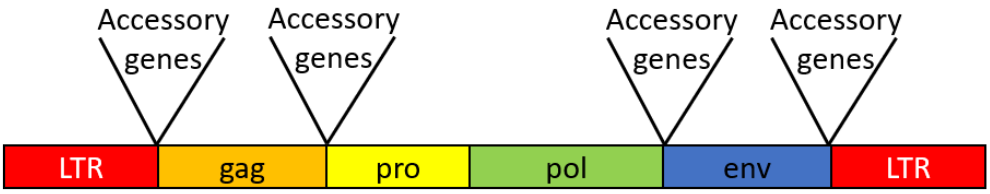
More than two thirds of the human genome consists of repetitive elements or repeat regions (de Koning *et al.*, 2011), with debate as to how much of this genomic sequence has function. Transposable elements (TEs), colloquially known as "jumping genes" are a major category of repeat regions that can move about both within and more rarely between genomes. Retrotransposons (Class I transposable elements) represent an important and biologically fascinating type of these mobile genetic elements and are known to generate insertion mutations, cause genomic instability, alter gene expression and contribute to genetic innovation (Cordaux & Batzer, 2009).

In humans and mice, retrotransposons and other repeat elements form a significant component of the transcriptome (Faulkner *et al.*, 2009). Despite their potentially deleterious effects, by increasing genetic diversity within a species they can increase adaptability under new selective pressures, as well as benefit the individual (Upton *et al.*, 2011). It is an exciting, complex and largely unknown involvement that they have had in the genomic changes that have shaped the sheep species and variety of breeds that we recognise today. The domestication of sheep since 11000 BP represents a crucial step in human civilisation and studies utilising endogenous retroviruses as genetic markers have been able to distinguish primitive breeds from modern ones which has helped preserve

rare gene pools as well as provided insight into the history of pastoralist societies (Chessa *et al.*, 2009).

Retroviruses are single stranded RNA viruses that encode the enzyme reverse transcriptase (RT) which converts RNA into DNA during the life cycle of the virus, which then becomes integrated into the host's genome (Temin & Mizutani, 1970), and they can be transformed into a retrotransposon if the domains that enable mobilisation are inactivated or deleted. Transcription and reverse transcription is shared by other viruses and with many moveable genetic elements such as retrotransposons, with over 10% of the human genome being composed of sequences resulting from reverse transcription (retrosequences) (Temin, 1989, Temin, 1992). The retroviridae family have a wide range of hosts, with extensive molecular and genomic diversity. Their genome structure is shown in Fig 3.8 (Gifford & Tristem, 2003).

Figure 3.8 Retrovirus genome structure
Simplified from (Gifford & Tristem, 2003) which shows the four essential components/sequence domains : 1) Gag, encoding the viral capsid, matrix and nucleocapsid proteins; 2) pro, encoding the viral protease; 3) pol, which encodes the enzymes reverse transcriptase and integrase; 4) env which encodes the surface and transmembrane glycoproteins. At either end of the DNA form of the retroviral genome are long terminal repeat (LTR) sequences. Accessory genes determine the complexity of the viral genome.



Retroviral infection involving somatic cells means that the virus can be transmitted to uninfected hosts as "exogenous" retroviruses. When germ cells are infected the viral genome can become part of the host genome as an "endogenous" retrovirus (ERV). If movement between cells is not permitted (not infectious), the insertion of new copies into the genome is restricted to that same cell, thus relying upon germ cell infection to enable vertical transmission by subsequent generations (Naville *et al.*, 2016).

Retrotransposons can be divided into three groups:

1) Long terminal repeats (LTRs) : If they have LTRs and encode reverse transcriptase (like retroviruses). This group employ a 'copy and paste' mechanism to transpose themselves.

2) Long interspersed nuclear elements (LINEs, L1s) : If they encode reverse transcriptase but lack LTRs and are transcribed by RNA polymerase II.

3) Short interspersed nuclear elements (SINEs) : If they do not encode reverse transcriptase and are transcribed by RNA polymerase III.

The bovine genome, like that of mice and humans has been found to contain many of the same eutherian mammal repeats, in addition to long interspersed nuclear element RTE (BovB) elements that have been suggested to derive horizontally from squamata and some areas of the genome, identified by repeat regions have defined both ancestral vs ruminant-specific genomic regions (Adelson *et al.*, 2009). In ruminant animals the most common SINE is Bov-A2, and it has been suggested that genomic instability in bovine genomes can produce extensive unequal crossing over of Bov-A2 and that repeated outbreeding may contribute to levels of polymorphism (Onami *et al.*, 2007). There is large variation of Bov-A2 sequences, partly due to site specific micro-recombination followed by gene conversion, and short cDNAs copied by the RT have been suspected of being involved in the somatic hypermutation (SHM) process in the hypervariable regions of immunoglobulin and major histocompatibility complex (MHC) genes. Bov-A2 has also been found in the non-coding regions of several genes responsive to environmental stress, and it has been suggested that this sequence may play a role in the post-transcriptional regulation of gene expression as the "core" sequence has been identified at high levels in lymphocytes only after their activation (Damiani *et al.*, 2008).

Genomes have evolved defence mechanisms from these mobile "retro" elements and retrotransposons. Cytosine methylation, small RNAs and apolipoprotein B mRNA editing enzyme, catalytic polypeptide family (APOBECs) may all play a role in protection against infectious retroviruses (Fanning, 2011). Most ERVs have accumulated mutations which leave them unable to produce viral particles but some have managed to keep their open reading frames (ORFs) and are utilised by the host to fulfil important biological functions, participating in gene regulation, cell growth and the immune response (Stoye, 2012). John

Bittner (Bittner, 1936) and Ludwik Gross (Gross, 1951) used mice to first show that retroviruses can cause neoplastic disease, and they are involved in many diseases such as cancers (leukaemias, mammary carcinomas), immunodeficiencies and arthritis (Vogt, 1997). The interesting exception is the Spumavirus class, which can persistently infect wild non-human primates, felines, bovines, equines and small ruminants, with no apparent pathological consequences (Murray *et al.*, 2008).

A preliminary investigation has focussed on exploring the LTR group, as this group is suspected of accounting for the significant number of reads that Kallisto2 was unable to pseudoalign to the reference transcriptome. Both retroviruses and long terminal repeat (LTR) retrotransposons are flanked by LTR regions in direct orientation that are necessary for transcription and integration into the genome, with the main difference between them being the presence of the envelope gene.

Repeat regions in the sheep genome were identified using the UCSC genome browser for OarV3.1. Within the toolset of UCSC, the tablebrowser function can select the group concerning all variation and repeats and then specifically the repeat masker track within the reference sheep genome, requesting the specific fields: genomic sequence name (genoName), the start in genomic sequence (genoStart), the end in genomic sequence (genoEnd), the relative orientation of the strand (+ or - strand), the name of repeat (repName), the class of repeat (repClass) and the family of repeat (repFamily). From this extensive list, 396,061 locations with the LTR class of repeat (Group 1 above) were selected and the co-ordinates extracted, producing regions that have been examined in more detail using the ribodepleted 0hr and 7hr data from each sheep. The locations mapping to scaffolds were ignored and the file formatted to produce a bed file (the start location for each region begins at 0 so each start location had -1 deducted) so BEDtools could be used to count the reads overlapping by >1bp to these regions of interest from the selected BAM files of interest (specifically the 0hr and 7hr total RNA samples from each individual). After collating all the counts for each set of co-ordinates for all 0hr and 7hr ribodepleted total RNA alignment files for each individual, the TPM for each co-ordinate were determined utilising the SAMtools flagstat statistic on how many reads were in that particular alignment file. Using the pivot table function in Excel, it was then possible to examine the various names of repeat (246) and families (6) of repeat, both across all individuals as a group and then separately.

As an LTR group, differences in proportions of raw reads mapping to regions of known ERVs as a whole between 0hr and 7hr was negligible, with a Welch two sample t-test identifying a P value of 0.451, and a paired t-test P value of 0.3302. Examining the LTR group across all individuals, there was individual variation in the proportions of reads mapping at 0hr LPS compared to 7hr LPS. 5 out of the 6 animals showed decreased mapping in response to LPS but Male 1 had a greater percentage of reads mapping at 0hr compared to 7hr LPS.

There was variation between the individual Classes of ERVs in response to LPS, with 5 out of the 6 Classes recognised as having decreased mapping in response to LPS, and only one class showing an increase in the 7hr compared to 0hr LPS. This sub class of ERV1 called "ERV1?" (so-named by NCBI) had significantly (Welch two sample t-test P value = 0.02579) more reads mapping either in response or as a consequence of the LPS response, despite being the smallest class, containing just 638 regions, compared to ERVL which contains 100,223 regions.

Examining the individual names of repeat region, the most regulated (down in response to LPS), with the greatest proportion of reads mapping (more than twice the 2nd most abundant region, MLT1D) was BTLTR1. BTLTR1 is a known ruminant specific LTR-containing repeat region (Elsik *et al.*, 2009) and it has been debated as to whether this short, shared sequence is functionally significant and resulted in the transfer from BovB which was transferred horizontally among reptiles, ticks and ruminants (Walsh *et al.*, 2013), to BTLTR1, as a region of sequence similarity has been previously identified between BovB and BTLTR1 (Adelson, 2008, Adelson *et al.*, 2009). BovB is known to have been incorporated into the ruminant ancestor genome <50 MYA which is thought to be recent compared to others inactive for >100 MYA such as LINE L2 and SINE MIR (Adelson *et al.*, 2009), and the horizontal transfer of BovB has been shown to have transformed many vertebrate genomes (Walsh *et al.*, 2013). The identification of considerable BTLTR1 mapping in sheep in this study provides compelling evidence that BTLTR1 is particularly relevant to genome evolution for the lineage leading to domestic sheep, and suggests future study of the LINEs (which includes BovB), may be productive.

The reads defined as mapping to each reference ERV region are not strand specific to the ERV region and there may be significantly differentially expressed genes that lie either within or in very close proximity to these regions, so it is possible that these are confusing the overall picture as reads have been included when they only have to overlap by <1bp.

3.7 Discussion

RNA-Sequencing, using both polyA-selected and ribodepleted library preparation methods for sheep BMDMs across an LPS time course has provided genome-wide transcriptional information regarding the dynamic changes of gene expression that take place following TLR4 activation. Using two independent pipelines, Kallisto and StringTie, raw sequence data has been processed which has provided evidence that sheep BMDMs share a very complex transcriptome with macrophages of other species. Sheep macrophages have been estimated to utilise over 82% of the reference transcriptome OarV3.1. StringTie identified more than three times the number of reference transcript models than are currently recognised, and with all sequence available to Ensembl, the coverage of the current reference genome OarV3.1 has been greatly improved and will contribute to improving future versions.

The advantage of RNA-Seq, lacking a saturation limit, has proved a frustrating double-edged sword. The phenomenal abundance of certain transcripts, which are not regulated, cell specific or of immediate interest (such as *SPP1*, identified in Figures 3.2.A.ii and 3.3.A.ii), clearly occupy the majority of precious and expensive read coverage at the cost of detection of the rarer transcripts, which may be of great biological importance (see Figure 3.1). The computational, algorithmic and logistical challenges of processing, storing and analysing the volume of data created has also been fully appreciated.

Two pipelines, Kallisto and StringTie were used to obtain expression data. Both pipelines have been compared and generally demonstrate a positive correlation, with outliers highlighting either problematic, highly complex areas of the genome (where Kallisto determined greater expression than StringTie) or genes with many more transcript models than are currently recognised in the reference OarV3.1.81 or where the reference transcript model is not the most highly expressed model identified (where StringTie determined greater expression than Kallisto). Both pipelines have complementary strengths regarding accuracy of expression at reference transcript level (Kallisto) and gene level (StringTie), with StringTie being more labour intensive and computationally demanding, yet providing detailed information regarding intron and exon boundaries and novel splice variants. These strengths and weaknesses complement each other in appreciating the global transcriptional response across the whole genome, as well as providing validation of expression estimates.

The two library type preparation methods for RNA-Sequencing (polyA-selected and ribodepleted total) have been compared. There is a positive correlation between expression estimates derived from each library type. As expected, the greater variety of transcript type was detected in the ribodepleted libraries (which were also sequenced at the greater depth >100M). This highlighted many of the non-polyadenylated histones that are present in these samples, that were not detected by the polyA-selected libraries.

Given the abundance of individual transcripts taking up such a large proportion of the reads, and the many other types of transcript detected in the ribodepleted total samples, the polyA-selected library types detected the same proportion of the protein-coding transcriptome as the ribodepleted libraries. This was despite the polyA-selected library samples being sequenced at a quarter of the depth as the ribodepleted samples. In principle, if the additional transcripts detected in the ribo-minus libraries form a constant proportion of the "excess" detection, the effect of library type can be removed with a simple correction; removing counts attributed to all of those transcripts from the estimate of total counts used as a baseline for expression estimates/read number. This correction has now been successfully applied across the entire sheep atlas (Bush *et al.*, 2017). For the purpose of this project, such a correction was unnecessary, since both methods were applied to the same samples (0 and 7 hours). Indeed, comparison of these two samples was used to develop and validate the correction approach.

The preliminary investigation into LTR retrotransposons identified the ruminant-specific repeat region BTLTR1 to be the repeat region which has the greatest proportion of reads to map across all individuals. As a class of repeat region, BTLTR1 was found to be down-regulated in response to LPS. Taken together this suggests that of all the LTR retrotransposons, BTLTR1 has had the greatest effect on sheep genome evolution. However, the similarity in sequence to the LINE BovB, highlights the need for continued analysis involving all classes of retrotransposon. It is also recognised that a complete reference genome is essential as there are still many scaffolds that contribute to OarV3.1, which necessitate the need for extra long read technology. When the next, most complete version of the sheep genome is released, future plans involve re-mapping all the samples used in this project and repeating this analysis, as the quality, volume and RNA-Sequence from each individual will always be available from the unprecedented resource that is The

Sheep Atlas (the genomes of each individual were also sequenced and the data is available (Clark *et al.*, 2017)).

Chapter 4 Identification and annotation of sheep macrophage transcripts using immune cell data from the wider Sheep Atlas

4.1 Introduction

The Hume laboratory in which this study was conducted, has a long term interest in transcriptional regulation in macrophages and identification of genes required for the survival, proliferation and differentiation of the macrophage (Hume *et al.*, 2016), as well as comparative transcriptomics of macrophages from multiple species (Kapetanovic *et al.*, 2012, Fairbairn *et al.*, 2013, Karagianni *et al.*, 2016). Mice and humans are known to differ in several aspects of innate and acquired immunity (Mestas & Hughes, 2004, Marr *et al.*, 2006). There has been ongoing debate as to the value of mouse models of macrophage biology and chronic inflammatory disease (Schroder *et al.*, 2012, Seok *et al.*, 2013, Takao & Miyakawa, 2015). There is an increasing interest in large animal models, where both the immune system and physiology more closely resemble that of humans (Kapetanovic *et al.*, 2012)(see Chapter 1).

Miru (now Graphia Pro) is a computational tool which enables visualisation of large correlation networks and was discussed in detail in chapter 2.10. It has been used to identify clusters of co-expressed genes in complex datasets from multiple cancers, tissues and isolated cell populations (Freeman *et al.*, 2012, Doig *et al.*, 2013, Hume *et al.*, 2013, Mabbott *et al.*, 2013, Clark *et al.*, 2017), and to dissect the time course of macrophage responses to agonists (Kapetanovic *et al.*, 2012, Raza *et al.*, 2014, Baillie *et al.*, 2017). For the prioritised clusters, transcripts were explored individually to assign functional annotation (see Chapter 2.11.1) to as many as possible.

The web based tool GATHER (a Gene Annotation Toool to Help Explain Relationships) (Chang & Nevins, 2006) improved understanding of the pathways, processes and relationships that are shared between the transcripts of a given cluster of interest. GATHER (Chang & Nevins, 2006) provided an initial overview of the biology identified in each cluster, in conjunction with information gathered for each transcript using GeneCards (Stelzer *et al.*,

2016). GATHER enabled each list of genes within a cluster to be analysed against a series of data sources including annotations from evolutionary homologs, 'interactome' or protein-protein interactions and literature resources which use gene-to-gene co-citation networks (Jenssen *et al.*, 2001, Hoffmann & Valencia, 2004, Rual *et al.*, 2005). This programme is based on human functional genomic data, so some gene members of the sheep clusters were excluded from analysis. Hence, it was regarded as a complementary tool and additional cited literature information from GeneCards for individual genes (pasted into Mcirosoft Excel) provided additional metadata to be scrutinised to provide improved understanding and an overview in terms of transcriptional regulation, protein complexes and relationships to known pathways for each cluster of interest.

Previous studies in the laboratory have analysed the gene expression profiles of pig macrophages, including bone marrow derived macrophages, monocyte derived macrophages and alveolar macrophages using microarrays (Freeman *et al.*, 2012, Kapetanovic *et al.*, 2013). Equine alveolar and peritoneal macrophages have also been compared with each other when exploring the importance of the local microenvironment (Karagianni *et al.*, 2016). This history provides a detailed framework on which to base methods (see chapter 2) and enable the sheep, a ruminant animal, to be compared to other species.

Resident tissue macrophages are situated within every mammalian tissue, forming up to 15% of the cellular content of any given tissue. As discussed in Chapter 1, macrophages in each tissue adapt to the local microenvironment, to perform specific functions (Chapter 1.3). Lineage-trace studies in mice indicate that the majority of tissue macrophages are seeded during embryonic development, and thereafter are capable of self-renewal (Guilliams *et al.*, 2013, Yona *et al.*, 2013, Ginhoux & Jung, 2014, Ginhoux & Guilliams, 2016). The major exception is the large macrophage population of the intestinal wall. Macrophages in the lamina propria of the gut are continually replaced by circulating monocytes and must selectively down-regulate pattern recognition receptors in order to maintain homeostasis and prevent chronic inflammation. Blood monocytes in turn, are derived from bone marrow and myeloid stem cells, with *CSF1R* encoding the receptor that controls macrophage differentiation (with two ligands: CSF1 and IL34) (Section 1.3). The macrophages of the gut wall are rapidly depleted following treatment of mice with anti-CSF1R antibody (MacDonald *et al.*, 2010, Guilliams *et al.*, 2013). Taking these findings

together, Baillie et al (Baillie *et al.*, 2017) have argued that monocyte differentiation in response to CSF1 provides a model for the differentiation of the macrophages of the lamina propria of the gut wall, where it is crucial that they down-modulate their response to the intestinal microflora to avoid chronic inflammation.

Macrophages of the lung, on the other hand, must provide a first line of defence against inhaled microorganisms. Comparative analysis of pig alveolar macrophages with monocyte derived and bone marrow derived macrophages grown in CSF1, revealed that the lung macrophages selectively up-regulated phagocytic receptors, including multiple C-type lectins, and pattern recognition receptors (Freeman *et al.*, 2012, Kapetanovic *et al.*, 2013).

In this Chapter, the aim is to systematically compare the expression profiles of BMDM with other macrophage populations and immune cell types within the wider atlas. This will identify transcripts that are either a) enriched in macrophages, b) induced in cells of the macrophage lineage or c) associated with tissue-specific adaptation and preferentially expressed in certain types of macrophage. This will highlight transcriptional signatures that sheep share with other species, but the main focus for discussion is on aspects that separate sheep from other species.

4.2 Methods

All available immune cell sample RNA seq from the Sheep Atlas were utilised, summarised in table 4.1. The raw RNA-Seq data was taken and processed in exactly the same way as that of the BMDM response samples (see chapter 2). The same pipelines, both Kallisto and StringTie, generated transcript and gene co-expression profiles respectively for all these immune cell samples in Table 4.1 (see Appendix 4.0). These expression estimates (transcript expression as TPM, using Kallisto, and gene expression as FPKM, using StringTie), were then examined using Miru. Clusters of transcripts that share an expression pattern common to all individuals, were extracted and examined more thoroughly.

Table 4.1. Table showing the samples from the Sheep Atlas which have been utilised in chapter 4. Where samples are missing from individual animals, there was not enough high quality RNA to be sent for sequencing, thus RNA seq data was not available. All samples utilised were sequenced as described in Chapter 2, using polyA-selection at >25M read depth.

 indicates polyA-selected libraries, >25M read depth, paired end RNA-Seq data.

Sheep Atlas animal	Female 1	Female 2	Female 3	Male 1	Male 2	Male 3
BMDM 0hr LPS						
BMDM 2hr LPS						
BMDM 4hr LPS						
BMDM 7hr LPS						
BMDM 24hr LPS						
Alveolar macrophages (AMs)	n/a	n/a	n/a		n/a	
Monocyte derived macrophages (MDMs)		n/a				
Blood Leuckocytes (BLs)	n/a		n/a		n/a	
Peripheral blood mononuclear cells (PBMCs)			n/a		n/a	

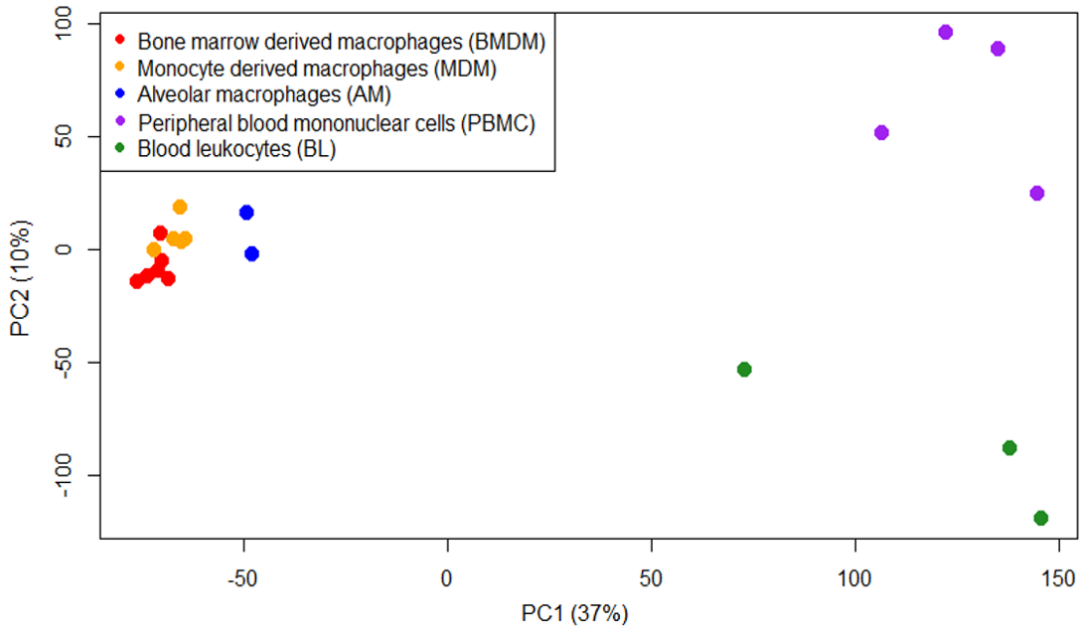
4.3 Results and Discussion

4.3.1 Examination of a principal components analysis of Sheep Atlas immune cells

A principal component analysis (PCA) biplot of the reference transcript expression (Kallisto, TPM) profiles for each individual immune sample is shown in Figure 4.1. As expected, the macrophages cluster together, whereas the peripheral blood leukocytes and peripheral blood mononuclear cells are clearly distinct from both the macrophages and each other. As observed in pigs, the BMDM and MDM have overlapping profiles, but the alveolar macrophages are much more similar to BMDM/MDM than they were in pigs (Kapetanovic

et al., 2013), perhaps reflecting that this analysis included both BLs and PBMCs as well as the macrophage samples.

Figure 4.1 Principal components of all Sheep Atlas immune cell samples
Each immune cell sample RNA-Seq data (polyA-selected 25M paired end reads) were processed through the Kallisto pipeline (See Chapter 2.8.1) and transcript expression estimates (TPM) for every reference transcript in Oar V3.1.81 were analysed across all samples. The resulting PCA biplot is coloured by cell type. The percentage of variance is in brackets for each principal component.



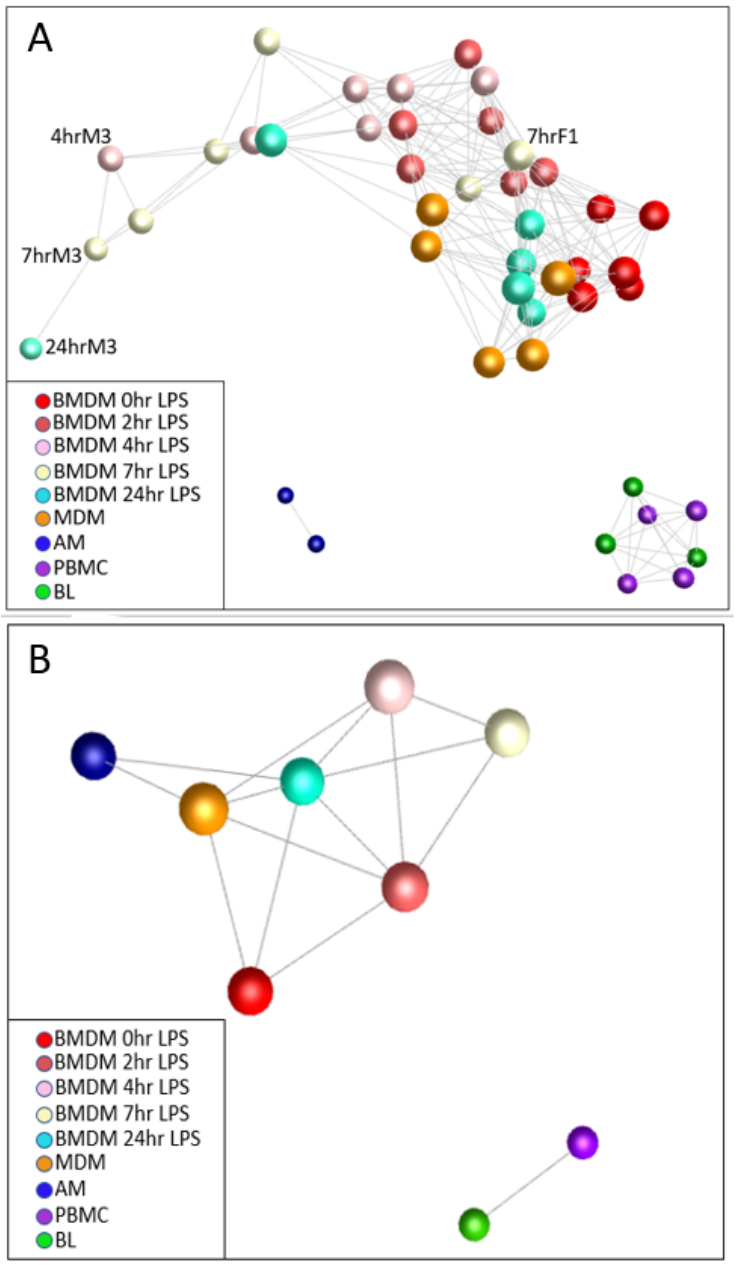
4.3.2 Network analysis

To identify genes expressed specifically in macrophages, or macrophage subpopulations, relative to other leukocytes, that provide the differential profiles evident in the PCA (Figure 4.1), I performed a network analysis using Miru. For the purpose of this analysis, only transcripts expressed above 5 TPM (deemed robust expression) in at least one sample were included, which reduced the dataset to a manageable size (13,812 transcripts) and reflects the cell-type specific transcriptome better that the more relaxed criterion. A transcript-to-transcript analysis of these data without filtering was dominated by clusters with low expression and/or individual-specific expression (not shown). A sample to sample analysis based on this set of transcripts across all individual immune cell samples (Figure 4.2A), was performed to analyse the relationships between samples. This created a network graph containing 44 nodes (a node for every immune cell sample) with 218 edges with a Pearson correlation coefficient of (R) = 0.92, to include all 44 nodes. An MCL inflation value of 2.2

(Miru's optimal default setting) created clusters of samples with highly correlated expression patterns. This network graph is broadly consistent with the PCA, in that the blood leukocyte and PBMC were clearly distinct, and the AM formed a separate cluster. The inclusion of the LPS-stimulated macrophages revealed the global change in profile in response to this stimulus, and also the distinctive response of one individual, Male 3 (M3 samples).

A stringent analysis of cell type relationships was therefore performed on aggregated data. The dataset was filtered further to remove transcripts that were not expressed at >30 TPM in at least one sample. The expression values for each remaining transcript (expressed >30tpm in at least one sample), were then averaged across all individuals for each immune cell type. The clustered sample-to-sample network graph contained 9 nodes (a node for each immune cell type) with 15 edges with a Pearson correlation coefficient of (R) = 0.82 and MCL inflation value of 2.2 to create Figure 4.2B. This analysis reduces the divergence between the macrophage samples, which form a single cluster distinct from the other leukocytes.

Figure 4.2 Sample-based networks of transcript expression
A From individual Sheep Atlas immune cell samples.
All samples which reached > 5 TPM in at least one sample were included. The Pearson correlation coefficient threshold was 0.92 and the MCL inflation value for clustering was 2.2. Each node (sphere) represents a sample and the lines between them represent correlations of at least 0.92. Nodes are coloured according to the sample type.
B From averaged transcript expression All transcripts which reached >30 TPM in at least one sample were averaged for each immune cell type, using all immune cell data from the Sheep Atlas Project. The Pearson correlation coefficient threshold was (R) =0.82, MCL inflation value was 2.2. Each node represents one cell type or treatment and the edges between them show correlations of > 0.82.



Using the same filtered list of transcripts, a transcript-transcript co-expression network graph, using a Pearson correlation coefficient of coefficient (R) = 0.9, containing 6,654 nodes with 1,210,700 edges, clustered with an MCL inflation value of 2.2 can be seen in Figure 4.3, which also highlights the position of key clusters which have been selected for futher analysis within the graph.

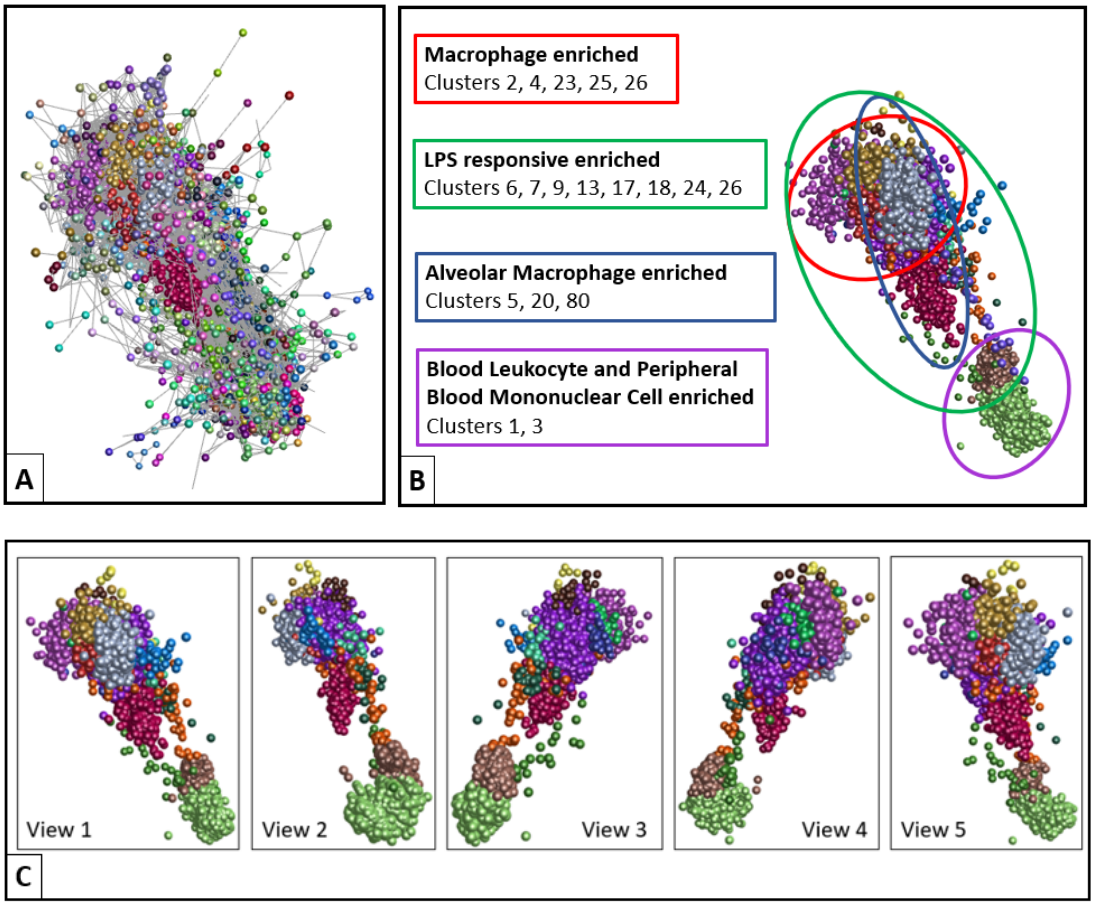
This transcript-transcript co-expression analysis revealed clusters of transcripts with enriched expression in specific populations of cells. All clusters were examined by looking at the mean associated expression pattern histogram and known genes within each cluster. The key clusters showing increased expression of transcripts within a given cell types of interest were extracted and grouped together according to which immune cell type they were enriched in, along with clusters of interest which contain genes of known function such as *CSF1R*. The transcripts within these key groups and clusters can be seen in the Excel workbook in Appendix 4.1. Ensembl Biomart (Aken *et al.*, 2016) was used to add metadata information for each ensemble transcript stable ID. The content of each of the clusters is examined in detail below.

Figure 4.3 A network layout based on average transcript expression across Sheep Atlas immune cells

A. The co-expression network layout, based on average expression across Sheep atlas immune cell types from transcripts expressed >30 TPM in any sample. The network contains 6654 nodes and 1210699 edges and was derived based on Pearson correlation coefficient >0.9. Nodes (spheres) represent transcripts and edges (connecting grey lines) represent the correlation between expression patterns of transcripts at a Pearson correlation coefficient of 0.9 or greater. Nodes of the same colour form a cluster.

B. An overview, highlighting positioning of key clusters, which share similar expression patterns that demonstrate specific increased expression of given immune cell lineages or stage of activation (LPS responsive). Nodes represent transcripts (averaged values). Edges have been removed for ease of visualisation but can be seen in 4.4.A. Minimum cluster size was 4 nodes and only those nodes that fell within a cluster are shown.

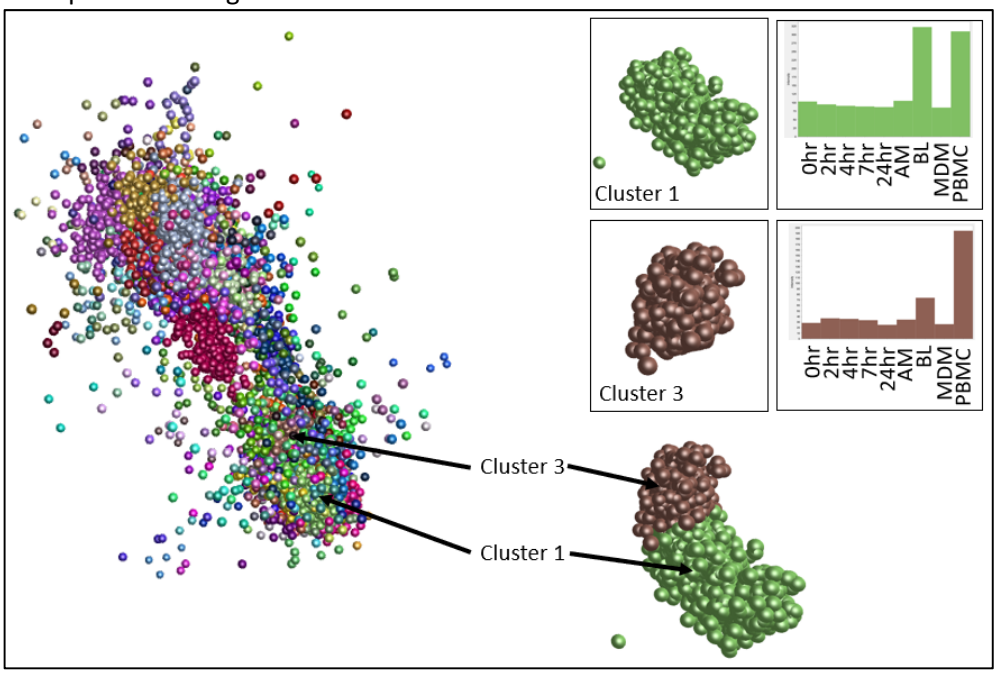
C. Selected clusters for analysis, containing genes with high expression in different sheep immune cells. These clusters of interest were extracted from the main network graph. Different views are shown, rotated clockwise 60^{o} from view 1.



Although the BM and PBMC clusters form a continuous network, the clusters are separated from both the general macrophage and the alveolar macrophage enriched clusters, as demonstrated in Figure 4.4.B which extracts the clusters of interest from this main network graph. The largest cluster 1 (pale green) and cluster 3 (pale brown), contain transcripts

which showed high expression in BLs and PBMCs (Figure 4.4), a total of 2215 transcripts. The two clusters are distinguished by relative enrichment in BLs. Cluster 3 is more mononuclear cell-specific but taken together these clusters were likely to contain genes enriched specifically in populations of blood lymphocytes, granulocytes and NK cells as opposed to macrophages. They may also contain monocyte-specific transcripts that are down-regulated with macrophage differentiation. Clearly, a much more refined analysis could be obtained by separating the populations of blood cells as in studies by the mouse ImmGen consortium (Heng & Painter, 2008) and in humans; examining peripheral blood leukocyte populations (B-cells, CD4+ T-cells, CD 8+ T-cells, lymphocytes and granulocytes) (Palmer *et al.*, 2006), and by the FANTOM5 consortium (Lizio *et al.*, 2015, Noguchi *et al.*, 2017). However, as a first pass, these clusters appear to contain transcripts that are enriched specifically in cells of the immune system. In the wider sheep transcriptional atlas thymus, spleen and multiple lymph node sample data is available (Clark *et al.*, 2017) so future studies could easily refine sheep B cell and T cell-specific clusters by including this data which has not been examined for this thesis.

Figure 4.4 Blood Leukocyte (BL) and peripheral blood mononuclear cell (PBMC) clusters extracted from the main network analysis, based on average expression across Sheep atlas immune cell types from transcripts expressed >30 TPM in any sample. Two particular clusters were enriched for transcripts expressed in PBMC and BLs: Cluster 1 (1686 Nodes/transcripts) and Cluster 3 (528 transcripts). The corresponding signal histograms showing the average expression of all the transcripts contained in each cluster, for each cell type can be seen next to the extracted cluster that is pictured alongside.



This analysis reveals transcripts that are not expressed by macrophages. Consistent with that proposition, these clusters contain the classic B lymphocyte cell related surface antigen genes found in mice and humans such as *CD19* which is indicative of mature -naïve B cells (Otero *et al.*, 2003) and the gene encoding a B-cell restricted molecule, *CD22 (Moyron-Quiroz et al., 2002)*. Other well documented B cell genes include *CD69* which is associated with B cell activation and *CD27*, indicating there are memory B cells and activation within these samples (Wei *et al.*, 2011, Kaminski *et al.*, 2012), and many of the B cell-associated genes identified in humans such as Major histocompatibility complex, class II, DM alpha (*HLA-DMA*), B-cell CLL-lymphoma 11A zinc finger protein (*BCL11A*) and transcription factor genes such as *IRF4, TCF4* and *TFEB* (Palmer *et al.*, 2006). The cluster also contains genes associated with T cells and T cell subsets (*CD3* and *CD8)*, and many known lymphocyte-associated transcriptional regulators, such as *LEF1*. The cytokine receptor gene *IL2RA* appears in this cluster and is known to be an early determinant of T lymphocyte fate specification (Chang *et al.*, 2014) and interestingly cluster 1 also contains *CNR2*, a gene known to encode a molecule with immunosuppressive effects in macrophages (Sacerdote *et al.*, 2000) and it has been suggested that this gene may play a role in maintaining homeostasis in naïve and memory T cells (Best *et al.*, 2013).

T cell: In common with ImmGen clustering examining T cell activation (Best *et al.*, 2013), there are also genes of the S1P receptor family and the prosurvival chemokine receptor *CX3CR1*, as well as the same members of E protein (class I) family of helix-loop-helix transcription factors (*TCF3* and *TCF4*) that have been identified which were identified as potential repressors of genes expressed in naïve and late effector memory T cell populations (Best *et al.*, 2013). The early NK cell activation markers *CD27* and the C-type lectin receptor *CD69* appear in cluster 3 but stem cell antigen 1 (*SCA1*) and killer cell lectin like receptor G1 (*KLRG1*), which have both been found to be dramatically increased with NK activation (Fogel *et al.*, 2013) are absent from these clusters.

Granulocyte: Multiple granulocyte-enriched genes identified in humans are found in these clusters such as colony stimulating factor 3 receptor (*CSF3R*), nuclear factor (erythroid-derived 2) (*NFE2*), fibrinogen-like 2 (*FGL2*), and the ets domain transcription factor E74-like factor 4 (*ELF4*) (Palmer *et al.*, 2006). The gene regulator of G-protein signalling 2 *(RGS2*), is also found in cluster 1, which has also been identified to be expressed in unstimulated circulating neutrophils (Bertrand *et al.*, 2004, Palmer *et al.*, 2006). None of the recognised

intracellular pathogen destruction genes, enriched in human granulocytes were present such as cathepsins B/ C/ S.

Somewhat less obvious or expected as they should have been depleted before the library preparation, was the finding that the most highly expressed, annotated transcripts within these clusters are ribosomal proteins (RPs) involved in assembly of small 40s ribosomal subunits: RPS8,11,19 and 21 and large 60s ribosomal subunits: RPLP0,1 and RPL8,29,32,34 and 37. These ribosomal phosphoproteins are probably involved in plasma cells which are protein secretion factories.

4.3.3 Identification and annotation of transcripts enriched in the sheep macrophage lineage.

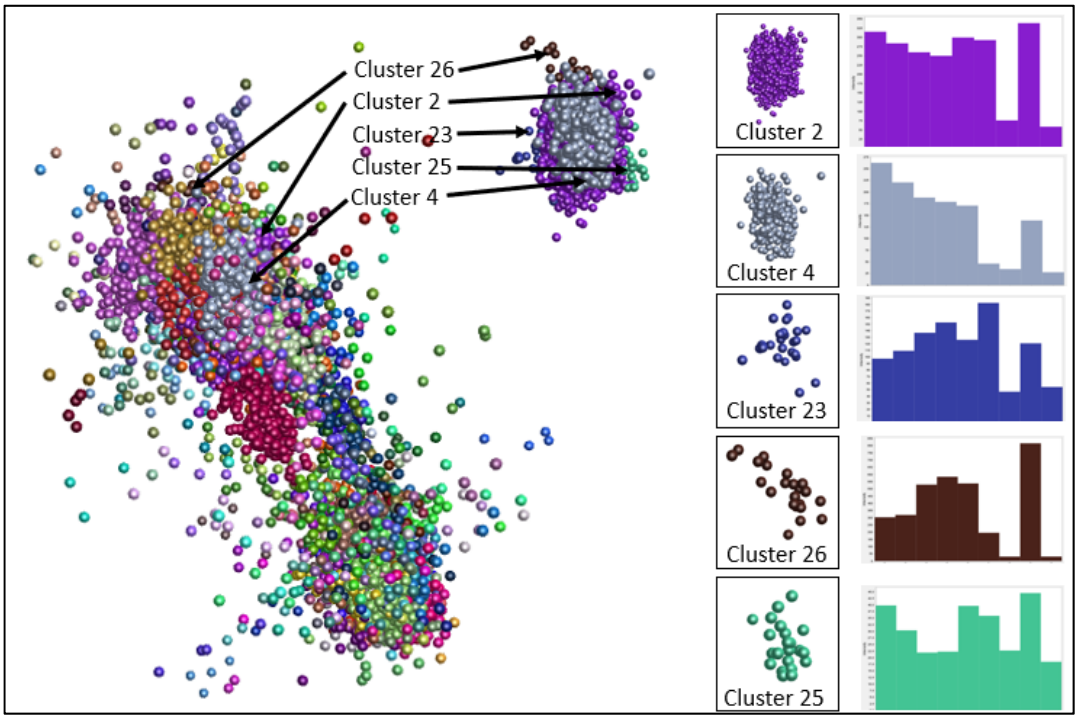
There are several clusters in which the average expression shows enrichment across all the macrophage samples, with low or absent expression within the BL or PBMC samples (Figure 4.5). The five clusters containing the greatest number of transcripts (2, 4, 23,25, 26) have been extracted and appear in Appendix 4.1, within the worksheet named BMDMs, AMs and MDMs.

In total, the five macrophage enriched clusters contain 1577 transcripts (with each transcript being from the reference transcriptome, having been assigned an expression estimate >30tpm by Kallisto2 in at least one immune cell sample). Based upon the averaged expression estimate for each cell type, the pattern of transcript expression across the different cell types was scrutinised using Miru. Each transcript has a potential function in innate immunity, and accordingly, each transcript that was identified only by an ENSEMBL ID, was subjected to manual annotation using ENSEMBL to examine the nearest known orthologues and validate by comparing regions for synteny. Genecards (Stelzer *et al.*, 2016) was used to check the validity of each gene that I had assigned functional annotation and determine the relevance and sense of its identification within that given cluster.

The clusters contain genes encoding numerous known transcriptional regulators implicated in lineage commitment including lymphocyte cytosolic protein 1 (LCP1), a plastin expressed only in haematopoietic lineages and malignant human cells, cellular repressor of E1A stimulated genes 1 (CREG1), which is known to regulate transcription of core macrophage-associated genes in the mouse (Gautier *et al.*, 2012), as well as MAFBZIP Transcription

factor B (MAFB), which I assigned to ENSOART00000001452/ ENSOARG00000001355, a bZIP transcription factor known to have an important role in the regulation of lineage-specific haematopoesis, and that recently has been shown to play a central role in in promoting cholesterol efflux and anti-inflammatory macrophage polarization (Kim, 2017).

Figure 4.5 Macrophage enriched clusters extracted from the main network analysis, based on average expression across Sheep atlas immune cell types from transcripts expressed >30 TPM in any sample. Five particular clusters were enriched for transcripts expressed across the BMDMs, AMs and MDMs. The corresponding signal histograms showing the average expression of all the transcripts contained in each cluster, for each cell type can be seen next to the extracted cluster that is pictured alongside. The columns representing immune cell types, appear in the following order from left to right: BMDM 0hr, BMDM LPS 2hr, BMDM LPS 4hr, BMDM LPS 7hr, BMDM LPS 24hr, AM, BL, MDM and PBMC.



Within these 5 macrophage clusters, 319 transcripts were identifiable only by ENSEMBL ID. Many of these were situated on scaffolds, with simple transcript models of only 1 or 2 exons and minimal information other than gene type. In total, for these 5 clusters, 138 ENSOARGs have been provided with a candidate orthology to a known gene, including several genes not previously associated with macrophage function; such as *COX7A2, WDR61, PGRMC1* and *SELENOP* (also known as *SEPP1* or *Selenoprotein P*).

SELENOP, found in cluster 4, is one of the most highly expressed transcripts, selectively within the sheep BMDMs and MDMs. *SELENOP* is widely-expressed in mice, albeit largely

absent from lymphocytes (www.biogps.org), whereas in humans, the transcript is enriched in liver, absent in monocytes and induced in MDM (Kawaji *et al.*, 2017). The relative abundance of this transcript seen here, most highly expressed in resting sheep macrophages, is consistent with the documented relative importance of selenium for innate immune function in sheep (Kiremidjian-Schumacher and Stotzky 1987, Dhur, Galan et al. 1990, Hall, Sendek et al. 2011, Hall, Vorachek et al. 2013) and this relative high expression might underpin the species differences seen in effects of selenium and vitamin E on the immune response of domestic animals (Finch and Turner 1996).

Clusters 2 and 4 are more specifically enriched in the BMDM and MDM samples. These cells share their exposure to CSF1 and extended *in vitro* culture, and co-segregate in the PCA (Figure 4.1), mirroring the sample to sample network analysis (Figure 4.2). The program and tool GATHER (Chang & Nevins, 2006) was used to help with understanding the known function of a given group of transcripts as it provides the most significant GO terms (Ashburner *et al.*, 2000). For these BMDM and MDM clusters, the most significant GO terms included G-protein coupled receptor protein signalling pathway, sensory perception, detection of external stimulus and cellular physiological process, reflecting the physiological phenotype of these cells. The larger cluster 2, contains 1247 macrophage lineage transcripts and many of the classic mature macrophage markers such as CD163 (a member of the scavenger receptor cysteine-rich superfamily), CD164 (a transmembrane sialomucin), CD14 (the coreceptor for LPS, see Chapter 1.4, along with many other genes known to be enriched in macrophages, such as phospholipase D3 (PLD3), tripeptidyl peptidase 1 (TPP1), lysosomal associated membrane protein 2 (LAMP2), transcobalamin 2 (TCN2), and ATP binding cassette subfamily A member 1 (ABCA1), present in these macrophage enriched clusters (Gautier *et al.*, 2012).

In other species, CSF1 promotes cellular proliferation and also induces a set of specific genes associated with tissue repair and lipid metabolism (Irvine *et al.*, 2009, Fairbairn *et al.*, 2011). Consistent with the shared cell proliferation, these macrophage enriched clusters contain numerous cyclin dependent kinase inhibitor genes such as *CDKN2C* and *CDKN3* as well as genes encoding components of the mitotic apparatus such as *BUB1B* and *CDC20.* Many transcripts seen here encode genes known to be involved in various cancers, including *CKS2* which is known to be related to pathways in cancer and small cell lung cancer (Valles *et al.*, 2012) and the important oncogene *BIRC5*, a multitasking protein that

has dual roles in promoting cell proliferation and preventing apoptosis, also found to enhance aerobic glycolysis and drug resistance (Hagenbuchner *et al.*, 2016).

Human and mouse macrophages grown in CSF1 differ in their gene expression profiles. In human MDMs, CSF1 promotes the expression of multiple genes involved in cholesterol metabolism and lipid accumulation, including *HMGCR, MVD, IDI1, FDPS, SQLE, CYP51A1, EBP, NSDHL, DHCR7,* and *DHCR24* (Irvine *et al.*, 2009). Of these genes, only *DHCR24* appears in this Cluster 2. However, also appearing in Cluster 2 is the ATP-binding cassette (ABC) transporter gene *ABCA1*, a cholesterol efflux pump in the cellular lipid removal pathway, *INSIG2* and *ELOVL1*. All these genes have been identified in mice macrophages and to be responsive to diet, being substantially upregulated in atherogenic diets compared to restrictive diets (Renaud *et al.*, 2014).

The long chain fatty acid translocase, encoded by *CD36*, was amongst the novel genes most highly expressed and annotated in the macrophage-enriched gene set in cluster 2 (assigned to *ENSOART00000018636/ ENSOARG00000017123*). This protein gene, along with *FABP7* (cluster 4) and *FABP5* (cluster 2), both of which are also highly expressed, are known to have a high specificity for long-chain fatty acids and other hydrophobic ligands (Vogel Hertzel & Bernlohr, 2000), and are involved in lipoprotein metabolism in humans and mice. Polymorphisms in *FABP5* are already known to be associated with type 2 diabetes (Bu *et al.*, 2011). This is of specific interest in humans because the upregulation of scavenger receptors capable of binding modified forms of LDL (such as CD36), are hallmarks of an imbalance in lipid metabolism and the formation of so-called macrophage foam cells (macrophages laden with cholesterol), which are present in atherosclerosis, and a result of apolipoprotein B in the subendothelial space causing local inflammation, the release of chemokines and chronic inflammation (Choudhury *et al.*, 2005, Moore *et al.*, 2013).

As mentioned in Chapter 2.4, Figure 2.1, the sheep macrophages grown in CSF1 do have a vacuolar appearance, which might be associated with formation of lipid droplets. Other very highly expressed transcripts found in cluster 4, which are involved in cholesterol metabolism are *LIPA,* the lysosomal acid lipase (also known as cholesteryl ester hydrolase), which separates cholesteryl esters into cholesterol and fatty acids (Brown & Goldstein, 1986) and *NPC2*, a gene encoding a protein known to bind unesterified cholesterol that has been released from LDLs, to regulate the transport of cholesterol through the late endosomal/lysosomal system (Frolov *et al.*, 2003). *PLD3*, the gene encoding phospholipase

D which is known to catalyse the hydrolysis of membrane phospholipids (Fazzari *et al.*, 2017), is also highly expressed and found in cluster 2.

Taken together, these results suggest that resting sheep macrophages grown in CSF1 are specifically adapted to deal with the accumulation of lipoproteins and cholesterol within the cell, resulting from the increased availability and uptake of long chain fatty acids in the normal healthy ruminant. Clearly regulation over some aspects of this gene expression profile are shared between sheep, mice and humans. Two of the ENSEMBL IDs, present in cluster 2 (*ENSOART00000001452/ ENSOARG00000001355* and *ENSOART00000003211/ ENSOARG00000002962*) have been assigned the functional annotation of the highly conserved transcription factors *MafB* and *XBP1* respectively, which are also important and well recognised in mouse and human macrophages (Martinon *et al.*, 2010, Kim, 2017), validating these manual annotations in sheep.

MAFB is a negative regulator of type I IFN and a pro-inflammatory cytokine, playing a pivotal role in regulating macrophage polarisation, and mediating anti-inflammatory and anti-atherogenic signalling in macrophages (Kim, 2017). MAFB regulates macrophage cholesterol efflux in mice by increasing expression of *ABCA1* (also in cluster 2), and is a downstream target of IL4/STAT6 (a STAT6 transcriptional binding site is conserved between mice and humans on the *MAFB* promotor), being strongly inhibited by proinflammatory signalling, remaining repressed in macrophage foam cells, thus it shares this same expression profile in sheep, being repressed in response to LPS (Kim, 2017).

The transcription factor XBP1 (assigned to *ENSOART00000003211/ ENSOARG00000002962*, also seen in cluster 2) in other species is involved in B cell differentiation and the production of immunoglobulins (Reimold *et al.*, 2001). XBP1 also promotes DC survival in a TLR ligand independent way and may be a therapeutic target for regulating the life span of DCs during an immune response (Leavy, 2007). There are multiple complex interactions between XBP1 and other transcription factors (Ozcan *et al.*, 2004, Ueki & Kadowaki, 2011, Chen *et al.*, 2014, Franca *et al.*, 2014, Kishino *et al.*, 2017). XBP1 activation through reactive oxygen species (ROS) production from TLR4 activation has also been shown to mediate the progression of steatosis (abnormal retention of lipids within a cell) to inflammation (Ye *et al.*, 2012) and this transcription factor has also provided the link between ER stress and intestinal inflammation (Kaser *et al.*, 2008). Given the importance and complexity of this transcription factor, future research is required to investigate the expression and regulation

of XBP1 in sheep compared to mice and humans. Improved understanding of the transcriptional regulation of these genes could provide novel management strategies for preventing atherosclerotic plaque development and promoting regression of existing atherosclerotic plaques in humans.

Macrophages are not restricted to their involvement in cholesterol homeostasis, but macrophages in the red pulp of the spleen, bone marrow and liver are involved in the removal of senescent red blood cells, the degradation of haem (a protoporphyrin IX ring surrounding an iron atom (Hamza & Dailey, 2012)), and recycling of iron for erythropoiesis (Korolnek & Hamza, 2015, Soares & Hamza, 2016, Theurl *et al.*, 2016). Free iron exchanges electrons with oxygen, catalysing the production reactive oxygen species (ROS) which are cytotoxic. However, iron is also essential for core biological functions, such as the production of ATP by the mitochondrial electron transport chain (Baranano *et al.*, 2000), and many haemoproteins (Kleingardner & Bren, 2015) play a central role in regulating macrophage function, therefore macrophage iron metabolism is tightly regulated.

Amongst the most highly expressed transcripts annotated in cluster 2, many are clearly associated with iron homeostasis. Ferritin heavy chain 1(*FTH1*), which was assigned to *ENSOART00000015613/ ENSOARG00000014339*), *FTH1-like* which was assigned to *ENSOART00000019236/ ENSOARG00000017679*) and ferritin light chain *(FTL*), which was assigned to *ENSOART00000013538/ ENSOARG00000012450*) are all enriched in the sheep macrophages. The gene zinc-iron permease *SLC39A1*, encoding a major endogenous uptake transporter, also appears in cluster 2 alongside the very highly expressed glutathione peroxidase 4 (*GPX4* which was assigned to *ENSOART00000012030/ ENSOARG00000011060* in cluster 2), and encodes an enzyme that protects cells against iron-dependent accumulation of lipid reactive oxygen species (Dixon *et al.*, 2012, Dixon & Stockwell, 2013, Cao & Dixon, 2016, Yang & Stockwell, 2016). Interestingly this isoenzyme is also a selenoprotein, underlining the importance of selenium in innate immunity in sheep (see Section 4.3.3). The high expression in these sheep macrophage enriched clusters suggests that sheep macrophages may be particularly susceptible to ferroptosis (non-apoptotic form of cell death due to iron dependent accumulation of lipid ROS and depletion of plasma membrane polyunsaturated fatty acids (Cao & Dixon, 2016)), and could provide a useful model to answering the many questions that still surround this cell death process that is linked to multiple human diseases (Feng & Stockwell, 2018). Multiple other

protective peroxidase genes are present in these macrophage clusters such as PRDX1, Peroxiredoxin 1 a thiol-specific peroxidase, and the relative high expression of these genes may suggest that redox reactions involving iron and lipid oxidation are mechanisms that sheep macrophages rely upon.

4.3.4 The sheep alveolar macrophage signature

Alveolar macrophages are positioned within the epithelium of the airways and alveoli, providing first line of defence against inhaled pathogens and surfactant homeostasis. Gibbings et al have discovered that the environment dictates the AM development, programming and function (Gibbings *et al.*, 2015), and as a result alveolar macrophages are highly adapted with unique characteristics to reflect their tissue location and function. Alveolar macrophages must not respond in a pro-inflammatory way to non-pathogenic antigens and are known to express a unique range of surface receptors and restrain from inflammation to ensure efficient gas exchange and close communication with alveolar epithelial cells (Hussell & Bell, 2014, Kopf *et al.*, 2014). Several studies in mice have documented the specific adaptation of tissue macrophages to specific environments (Gautier *et al.*, 2012, Mass *et al.*, 2016). Pig alveolar macrophages have been profiled as part of the pig atlas (Freeman *et al.*, 2012, Kapetanovic *et al.*, 2013) and stimulated with LPS/IFN-γ and IL4 to induce polarisation, where it has been shown to affect virus (PRRSV) replication (Wang *et al.*, 2017).

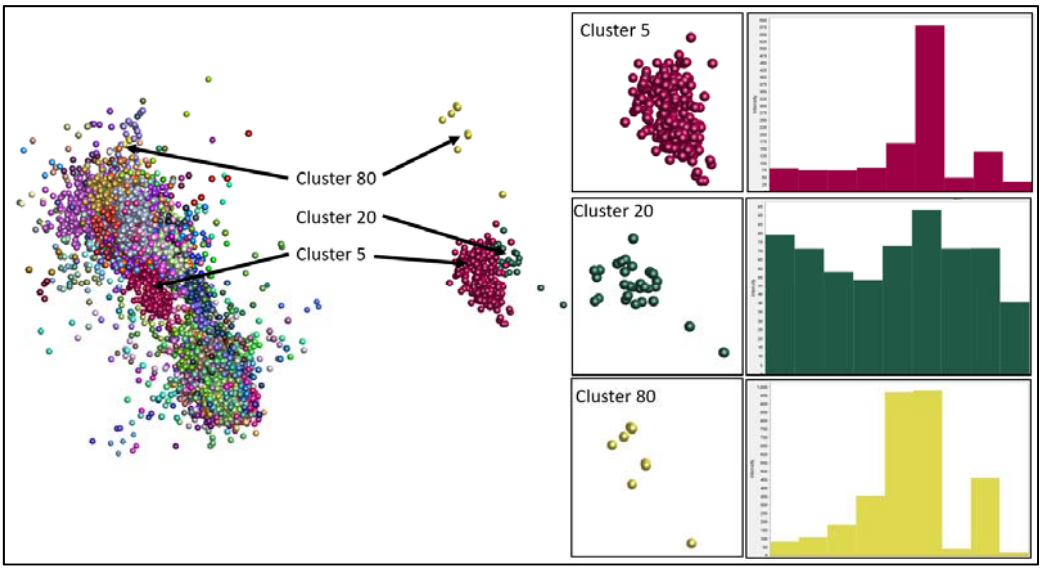
Alveolar macrophages have long been known to self-renew under normal steady-state conditions in the adult (Tarling *et al.*, 1987), having been seeded during waves of embryonic haematopoiesis, with monocytes only replenishing the resident alveolar macrophage population to a certain extent (Kopf *et al.*, 2014). Alveolar macrophages depend upon GM-CSF rather than CSF1 for their survival, proliferation and terminal differentiation (Shibata *et al.*, 2001). Horse (Karagianni *et al.*, 2016), mouse (Heng & Painter, 2008), pig (Kapetanovic *et al.*, 2013) and human (Xue *et al.*, 2014) alveolar macrophages have been profiled.

Several vital ruminant-specific processes that surround foregut fermentation are likely to impact upon the AM environment (Khadom *et al.*, 1985). Rumination requires regurgitation of rumen boluses of semi fermented complex carbohydrates, which are saturated with fermentative microbial flora (Braun *et al.*, 2013). The remastication and re-ensalivation of the bolus are vital processes that increase the volume of saliva (containing buffering phosphate to maintain rumen pH) produced (Kay, 1966). Lastly there is the

ruminant specific process of eructation, to rid the rumen of the huge volume of gas produced through fermentation (90% methane, 10% carbon dioxide) (Dougherty, 1968). It is likely that the pharynx and glottis fail in preventing any contamination of these products from entering the lungs, so it is inevitable that the ruminant alveolar macrophage will be frequently exposed to a range of substances that simple stomached animals are not.

The PCA and network analysis above indicated that sheep AM were not greatly different to BMDM and MDM. There were, nevertheless, several clusters of transcripts that were enriched in AM (Figure 4.6).

Figure 4.6 Alveolar macrophage enriched clusters extracted from the main network analysis, based on average expression across Sheep atlas immune cell types from transcripts expressed >30 TPM in any sample. Three particular clusters were enriched for transcripts expressed in AMs. The corresponding signal histograms showing the average expression of all the transcripts contained in each cluster, for each cell type can be seen next to the extracted cluster that is pictured alongside. The columns representing immune cell types, appear in the following order from left to right: BMDM 0hr, BMDM 2hr, BMDM 4hr, BMDM 7hr, BMDM 24hr, AM, BL, MDM and PBMC.



Cluster 5 is the largest, most AM-enriched; Cluster 20 is enriched in alveolar macrophages that also appear to be downregulated in response to LPS; Cluster 80 is enriched in alveolar macrophages and transcripts that also appear to be increased in expression in response to LPS.

Using GATHER to examine the 230 transcripts within cluster 5, the top GO terms are cell communication, signal transduction, response to biotic stimulus, organismal physiological process, immune response, defense response, sensory perception of chemical stimulus and

response to pest, pathogen or parasite, all of which underline the expected functional phenotype of these cells.

The list of transcripts in cluster 5 that are specifically enriched in sheep AM appears to lack many of the transcripts that discriminate AM from other macrophages that have been recognised in mice or pigs (Shibata *et al.*, 2001, Gautier *et al.*, 2012, Kapetanovic *et al.*, 2013, Misharin *et al.*, 2013, Xue *et al.*, 2014). No genes encoding the classic specific AM markers such as *SIGLECF, CD163*, or the GM-CSF receptor (*CSF2RA*) appear to be selectively expressed by sheep AM, using these MCL clustering parameters. All these genes were robustly expressed in the sheep AMs, as are both transcript variants for the transcription factor, peroxisome proliferator-activated receptor gamma (*PPARG*), encoding a nuclear receptor that regulates peroxisomal beta-oxidation of fatty acids and determine the differentiation and identity of AMs (Schneider *et al.*, 2014).

The transcript encoding a fundamentally important transcription factor, Hypoxia-inducible factor (*HIF1A*) appears in cluster 5. This transcription factor enables organisms to adapt to changes in oxygen tension (Semenza *et al.*, 1994). There is extensive literature on HIF1A in macrophages. Recent evidence connects HIF1A to LPS inducible changes in metabolism (Palsson-McDermott *et al.*, 2015). It may be that constitutive expression in sheep AMs supports a response to changes in oxygen tension in the lung. Cluster 5 also contains the transcript encoding transforming growth factor (TGF) beta receptor 2 *(TGFBR2)*. TGFBR2 binds the cytokine TGF-beta, which is also known to promote the development and homeostasis of AMs (Yu *et al.*, 2017). Mannose-6 -phosphate receptor tyrosine kinase insulin like growth factor 2 receptor gene *(IGF2R)*, which encodes a receptor known to take up lysosomal enzymes (Kornfeld, 1992, Kang *et al.*, 1998) also appears in this AM enriched cluster 5.

Sheep AMs also appear to express the gene encoding paired box 2 *(PAX2),* seen in cluster 5. *PAX2* encodes a multidomain transcription factor involved in kidney differentiation (Barua *et al.*, 2014) and the development of the urogenital tract, eyes and CNS. Within the wider atlas, *PAX2* appears most highly expressed in these tissues in addition to the alveolar macrophages, suggesting a sheep AM specific novel requirement of this transcription factor.

Both reference transcripts for the C type lectin domain containing 7A *(CLEC7A)*, which encodes dectin-1, are seen in cluster 5. Dectin-1 is the myeloid pattern recognition

receptor for beta-glucan polysaccharides that are derived from fungal cell walls and is required for effective immune response to fungi, even in the presence of opsonins (Taylor *et al.*, 2007, Vautier *et al.*, 2012). This highlights the danger posed from ruminal fungal populations burdening the ruminant lung and risk of fungal dissemination. Dectin-1 has been found to have an important role in non-pathogen mediated inflammation, with ligation in macrophages driving immune suppressive cellular differentiation (Daley *et al.*, 2017), which supports the requirement for ruminant AMs to reduce any pro-inflammatory responsiveness to stimuli, and induce immune suppression in response to stimuli.

The highest expressed transcripts enriched in alveolar macrophages were from genes encoding members of the S100/calgranulin protein family. These calcium binding cytosolic proteins are involved in many intracellular and extracellular functions (Marenholz *et al.*, 2004, Donato *et al.*, 2013, Gross *et al.*, 2014, Xia *et al.*, 2017). The genes *S100A12* and *S100A8* appear in cluster 5, and *S100A9* appears in cluster 80. S100A8 and S100A9 are known to form a complex and all three of these myeloid related proteins are released in response to stress and inflammation and to mediate responses through activating the cell surface receptors RAGE, TLR4, CD147 or GPCR which influence cell differentiation, inflammatory cell migration, cell apoptosis, cell proliferation, inflammatory cytokine production and tissue repair in macrophages (Foell *et al.*, 2004, Xia *et al.*, 2017). They can be used as biomarkers associated with certain diseases, with *S100A12, S100A8, S100A9* (and *S100B,* which is also in cluster 5 but not as highly expressed as the others), being linked to auto-inflammatory diseases, stroke and trauma (Basta *et al.*, 2006, Nakashima *et al.*, 2010, Zhao *et al.*, 2013, Yayar *et al.*, 2015). The high expression of these transcripts in AM is shared with pigs (Kapetanovic *et al.*, 2013).

Extracellular S100A8, S100A9 and S100A12 all mediate vascular inflammation through the RAGE receptor which is a multi-ligand receptor for advanced glycation end products which results in a cascade of transcription involving NFKB. This is particularly relevant humans, during diabetes and hyperglycaemia (Yao & Brownlee, 2010). It is thought that these proteins therefore mediate a further increase the generation of proinflammatory, prothrombotic and ROS molecules, exaggerating cellular damage even more (Goyette *et al.*, 2009, Yan *et al.*, 2010). *S100A12* was the most highly expressed transcript specifically in the sheep AMs and S100A12 is known to be granulocyte-specific and able to act independently of S100A8 and S100A9 during calcium-dependent signalling.

Mice have no homolog for the gene encoding S100A12 but, when transgenic mice had smooth muscle cell targeted expression of S100A12, they demonstrated increased coronary and aortic calcification and were more vulnerable to plaques (Hofmann Bowman *et al.*, 2011). S100A8 KO mice are embryonic lethal, however S100A9 KO mice are protected against mortality induced by endotoxic shock which suggested a detrimental role of these proteins during systemic infection and inflammation (Vogl *et al.*, 2007). The high expression seen in sheep AMs is contradictory to this, highlighting differences between sheep and mice and the dangers of relying upon mice to provide a model for human medicine. Using Ensembl to compare the regions between humans and sheep, sheep have identical synteny with humans, with the regions containing all the S100 genes in close proximity to one another and appearing identical on Chromosome 1 (Aken *et al.*, 2016). Taken together this suggests that sheep are more likely to resemble humans in the transcriptional regulation and control of expression of these genes and would provide a much better model for improving understanding and extrapolating their functional importance in innate immunity in future.

With respect to the lung, S100A12, S100A8/A9 are all significantly increased in human lung disease (Lorenz *et al.*, 2008). Increased levels of S100A12 relative to the heterodimer S100A8/A9, are characteristic of respiratory diseases with neutrophilic inflammation, with neutrophils known to be the first cells to migrate into the pulmonary compartment (Nauseef & Borregaard, 2014, Cantin *et al.*, 2015). The S100A8/A9 heterodimer has potent chemoattractant capabilities, mediating neutrophil influx following LPS stimulation in mice (Vandal *et al.*, 2003). The differential expression of high S100A12 relative to S100A8/A9 can distinguish acute respiratory distress syndrome from chronic disease such as cystic fibrosis and chronic obstructive lung disease, with the ratio increasing during times of acute exacerbations in chronic disease patients (Lorenz *et al.*, 2008).

Many transcripts in sheep macrophage clusters discussed previously highlight an importance for metal ions and amongst the most highly expressed transcripts in cluster 5 are those encoding genes for metal ion transporters. *TFRC (Transferrin receptor)* which is known encode a receptor responsible for cellular uptake of iron, is highly expressed as is *SLC31A1*, encoding a high affinity copper transporter and the paralog *SLC31A2*, encoding a copper transporter, all of which appear in cluster 5. The known macrophage specific gene *SLC11A1* encoding a protein involved in iron and manganese metabolism (NRAMP1), which

is associated with resistance to many intracellular pathogens (such as tuberculosis and leprosy) is also present in this cluster (Gruenheid *et al.*, 1997, Biggs *et al.*, 2001, Forbes & Gros, 2001, Forbes & Gros, 2003, Fritsche *et al.*, 2007). This suggests that sheep AMs are utilising the sequestration of metal ions to protect themselves from their own generation of ROS as well as effectively denying cations to engulfed pathogens.

Also amongst the top 10 most highly expressed transcripts of cluster 5 is *ENSOART00000022207/ ENSOARG00000020393* which has been annotated as Lysozyme-like. Examination of ENSEMBL indicates a genuine duplication of *LYZ* that is shared with cows and goats. Mice also have a duplication of *lysozyme*; one form *LYZM* is expressed by macrophages and the other, *LYZP* by Paneth cells in the gut. Lysozyme is known to be bacteriolytic and enhance the activity of immunoagents (Ragland & Criss, 2017), suggesting that ruminant macrophages have evolved to utilise this effective means of intracellular killing without triggering inflammation. It has been shown that the respiratory tracts of healthy sheep have dramatically different bacterial communities, dependent upon the depth within the lung, with fewer microbes the further distal from the glottis (Glendinning *et al.*, 2016). Taken together, this suggests that sheep AMs are highly effective in providing surveillance and clearance of pathogens and have harnessed useful mechanisms to kill whilst holding back the pro-inflammatory response.

In summary, it seems that AM in sheep have distinct transcriptional profiles from those of pigs and mice, utilising different transcription factors, and their responsiveness to microbial stimuli may be somewhat attenuated to avoid excessive response and inflammation to inhaled micro-organisms. The expression of the S100/calgranulin protein genes suggests that they (especially *S100A12)* may be conferring supportive mediation of innate immunity, indicative of innate immune response in the face of hyperlipidaemia and increased metal ion sequestration.

4.4 Conclusion

A great evolutionary biologist Dobzhansky wrote an essay “Nothing in Biology makes sense except in the light of evolution” (Dobzhansky, 1973). Clearly the evolution of the rumen and increased circulating long chain fatty acids and lipids and associated metabolic adaptations of sheep, have had an impact on the genes that are expressed by their immune cells.

Many highly conserved cellular markers (such as *CD163, CD14*, and *CD36* which was assigned to *ENSOARG00000017123*) and transcription factors (*HIF1A, MAFB* (assigned to *ENSOARG00000001355*) and *XBP1* (assigned to *ENSOARG00000002962*)), have been identified. For example, the very high expression of *S100A12* relative to *S100A8* and *S100A9* in the AMs has supported the view that *S100A12* is protective/ supportive of macrophage surveillance and stimulation without inducing proinflammatory cytokine production as opposed to the suggestion that all these calgranulins perpetuate pro-inflammation in the face of atherosclerosis in humans. Improved understanding of the gene expression signature in healthy sheep macrophages has not only improved functional annotation of the reference genome but has revealed mechanisms by which the innate immune system of sheep harnesses the abundance of lipid and restrains these environmental conditions from leading to systemic inflammation, as is the case in monogastric animals.

Chapter 5 Detailed analysis of the sheep BMDM response to LPS

5.1 Introduction

Chapter 4 of this thesis focussed on the identification of transcripts enriched in resting sheep macrophages both in culture (BMDM and MDM) and *in situ* (AM). This chapter analyses the transcriptomics of the response of BMDM to the well-defined TLR4 agonist, LPS. The interaction between macrophages and LPS elicits a temporal cascade of gene expression changes, which has been analysed in multiple species including mice (Schroder *et al.*, 2012, Raza *et al.*, 2014), humans (Baillie *et al.*, 2017) and pigs (Kapetanovic *et al.*, 2012). On the basis of those studies, and to enable comparison to the published data in other species, time points chosen were 0hr (unstimulated), 2hr (direct early response), 4hr (mid early response), 7hr (peak inflammatory response) and 24 hr (resolution phase of response) (Sweet & Hume, 1996, Hume *et al.*, 2001, Nilsson *et al.*, 2006, Schroder *et al.*, 2012, Baillie *et al.*, 2017). Other members of the laboratory have used the same time points for studies in goat, cattle, water buffalo, rat, horse and chicken macrophages, enabling a global analysis of species diversity in the response (unpublished). Genes associated with immunity are under strong evolutionary selection. The underlying hypothesis is that novel mechanisms and patterns of gene expression have evolved that distinguish sheep from other species. Some of these unique features might be shared with other ruminants which also share exposure to rumen microorganisms and dietary inputs.

The signalling pathway triggered by TLR activation is described in detail in the introduction (Section 1.2.2). The optimal interaction between LPS and TLR4 involves three proteins, LPS binding protein (LBP), CD14 and MD-2 (see Section 1.5). The recruitment of distinct adaptors activates two separable effector pathways; 1) TIRAP/MyD88-dependent pathway, responsible for pro-inflammatory cytokine expression and 2) the TRAM/TRIF MyD88-independent pathway signalling, responsible for expression of Type I interferons and IFN inducible genes (Lu *et al.*, 2008, Hume, 2015) (Section 1.5). Inducible feedback regulation of this signalling involves dissociation of the adaptor complexes, and expression of inhibitory regulators that impact at every level of the signalling cascade (Kondo *et al.*, 2012, Anwar *et al.*, 2013, Baillie *et al.*, 2017). The response to LPS shows considerable evidence of variation between individual humans (Fairfax *et al.*, 2014) and inbred mouse strains (Raza *et al.*, 2014). Accordingly, this project compared six individual animals, looking for

both common pathways shared by all animals and differences between individuals. The animals were a first cross between two disparate breeds of sheep, the Scottish blackface and Texel, both of which will have been subjected to divergent selection for different traits (see Section 1.8). This intent was to maximise heterozygosity and therefore the numbers of expressed biallelic SNV variants to enable detection of differential expression of the parental alleles in RNA-Seq data and it is accepted that this strategy reduces the power of expression-based analyses such as RNA-Seq. Examination of allele specific expression is beyond the scope of this thesis but future plans involving these investigations will be discussed further in Chapter 6.

Activation of mouse macrophages elicits changes in glucose, glutamine and fatty acid metabolism that meet the energy requirements of the cell, but also lead to accumulation of metabolic intermediates with signalling functions (Biswas & Mantovani, 2012, El Kasmi & Stenmark, 2015, Kelly & O'Neill, 2015, Baseler *et al.*, 2016, Mills & O'Neill, 2016, Langston *et al.*, 2017, Mazzone *et al.*, 2017).

It is unclear whether these metabolic responses are species-specific. Many LPS-inducible gene expression changes are not conserved (Schroder *et al.*, 2012). The Hume lab has identified conserved and divergent features of the LPS response of macrophages of mice, humans and pigs under comparable *ex vivo* conditions (Kapetanovic *et al.*, 2012, Schroder *et al.*, 2012, Kapetanovic *et al.*, 2013, Baillie *et al.*, 2017). In this chapter the temporal response of sheep BMDM has been examined in detail to improve understanding of innate immunity in sheep as a species and to identify features that may distinguish sheep from monogastric species. The gene expression patterns from individual sheep have been examined to determine regulated genes that vary most in their levels of expression between individuals at each time point.

5.2 Methods

The methods for macrophage cell culture, LPS stimulation, mRNA isolation and RNA-Seq were described in Chapter 2. The raw RNA-Seq data was processed in two pipelines: Kallisto (described in Chapter 2.10) used the available reference OarV3.1.81 transcriptome. The StringTie pipeline provides detailed exon level co-expression data for every known reference transcript and identifies completely novel transcript models, that are absent from OarV3.1, which was important as the reference OarV3.1 is not a complete or perfect transcript library.

Expression estimates from each pipeline were analysed using the network-based cluster analysis tool Miru (described in detail in Chapter 2.9). Clusters with informative average profiles (e.g. induced with a peak at a defined time) were prioritised for further annotation. Within those prioritised clusters, index genes of known function (e.g. inflammatory cytokines, transcription factors) were associated with genes that currently lacked functional annotation.

Within priority clusters, associated q values from statistical packages were assigned to each transcript. Transcripts that were expressed above a minimal threshold and significantly regulated at any time point were curated manually. Where possible they were assigned a functional name based on their nearest orthologues and conserved synteny with known genes in other species. To improve understanding of the pathways, processes and relationships that were shared between the transcripts of each cluster, the web-based tool GATHER (Chang & Nevins, 2006) (see Chapter 4.1), was utilised, in conjunction with information gathered for each gene using GeneCards (Stelzer *et al.*, 2016).

The sheep BMDM response data was examined within four different network expression graphs generated using Miru:

1) The Sheep Atlas (Clark *et al.*, 2017) network expression graph, shows the BMDM response-specific clusters 45 and 52. These clusters extract transcripts specifically expressed in response to LPS and enriched in stimulated macrophages relative to all other tissues. (See Appendix 5.1)

2) The Immune cell sample network expression graph (See Section 4.3.2 for methods), extracts the BMDM response-specific clusters (See Section 5.3.3 for results and Appendix 4.1 sheet 4) and identifies the set of transcripts that is macrophage and LPS response-specific relative to other immune cells.

3) The BMDM response for individual sheep sample network expression graph using Kallisto expression estimates based on the OarV3.1.81 reference transcriptome (See Section 5.2.1 for methods and 5.3.4 for results, Appendix 5.2. P and q values were obtained using Sleuth), highlights the temporal response that is shared by all individuals, as well as clusters demonstrating individuals to vary in their response.

4) The BMDM response for individual sheep sample network expression graph using StringTie expression estimates (See Section 5.2.2 for methods and 5.3.4 for results,

Appendix 5.3. P and q values were obtained using Ballgown), highlights gene expression patterns of the temporal response, including novel transcript models and genes, that are shared by all individuals, as well as clusters demonstrating individuals to vary in their response.

5.2.1 Establishing a sample network expression graph using Kallisto expression estimates for individual sheep BMDM responses

The expression estimates across all OarV3.1.81 transcripts, derived from polyA-selected libraries from BMDM at each time point, were collated together into a single table (Appendix 5.4). The maximum and minimum expression estimates along with the sample from which that maximum or minimum value was derived are shown and the ratio ((Maximum +1)/(Minimum +1)-1) was also calculated for every transcript. The table was filtered to exclude any transcript with a maximum expression estimate < 1 TPM and a ratio < 1.5. This filtered dataset (Appendix 5.6, the input file for Miru) contained 15,212 transcripts that were then clustered using Miru with a Pearson correlation r=0.95, MCL = 2.2, with 10,304 nodes and 360,840 edges in 284 clusters containing more than 3 nodes (Appendix 5.7). 3,898 nodes, where the expression profile did not resemble that of other transcripts sufficiently to create a cluster, were grouped together by the program as 'no class'.

5.2.2 Expression estimates for individual sheep BMDM responses using StringTie

All results from StringTie can be found in Appendix 5.6. The StringTie pipeline identified 76,411 transcripts expressed (FPKM>0) at some point over the LPS response. A total of 20,196 of these are reference transcript models, 42,154 novel transcript models to known genes and 14,061 novel transcript models associated with novel genes. Transcript variants of the same gene were grouped together resulting in 32,615 expressed genes (FPKM>0 in any LPS response sample), which includes all reference transcripts and novel gene transcript models derived by StringTie. The maximum and minimum expression estimates (FPKM) and expression ratio ((Maximum +1)/(Minimum +1)-1) was calculated for every gene. The table was then filtered to exclude any gene with a maximum expression estimate < 5 FPKM or an expression ratio < 1.5. This filtered dataset (Appendix 5.8) containing 10,552 transcripts were then clustered using Miru, with a Pearson correlation r=0.85, MCL = 2.2, resulting in 9,674 nodes (genes) and 938,068 edges in 281 clusters, of at least 3 nodes (Appendix 5.9).

5.3 Results

5.3.1 Overview of the sheep temporal LPS response

The expression estimates obtained for each reference transcript using Kallisto are contained in Appendix 5.4 and for StringTie in Appendix 5.6

Table 5.1 summarises the proportions of different transcript types that were detected over the BMDM time course and the transcriptional diversity of the sheep BMDM response. Nearly 90% of all nuclear-encoded OarV3.1.81 protein coding transcripts were detected in at least one BMDM sample. Even taking an arbitrary expression threshold of TPM >1, 85% of the reference transcriptome was detectable.

Table 5.1: Transcript types utilised over the sheep BMDM response to LPS
Biomart was used to determine the transcript type and Kallisto was used to estimate expression level. All transcripts demonstrating a maximum TPM > 0 across all polyA-selected LPS response samples have been included. The 'Mt RNA' transcript type includes both Mt rRNA and Mt tRNA. 'Pseudogenes' transcript type includes processed-pseudogenes and other pseudogenes.

Transcript Type	Transcripts expressed over LPS response	Reference Transcriptome	Percentage (%) OarV3.1
lincRNA	1,609	2,020	80
miRNA	817	1,305	63
Mt_RNA	20	24	83
Pseudogenes	229	290	79
protein_coding	20,503	22,823	90
rRNA	141	305	46
snoRNA	542	756	72
snRNA	594	1,234	48
	Total 24,455	**Total 28,757**	**85**

Table 5.2 Protein coding and total number of transcripts (OarV3.1.81) detected at each BMDM time point following LPS stimulation
Examining Kallisto expression estimates from the six adult sheep for each time point and extracting the number of OarV3.1 protein coding and total number of transcripts demonstrating a TPM > 0 in at least one sample.

LPS response timepoint	0hr	2hr	4hr	7hr	24hr
Protein coding transcripts detected across all individuals	18,270	18,170	18,053	18,321	18,260
Total number of OarV3.1.81 Transcripts detected across all individuals	**21,032**	**20,928**	**20,778**	**21,130**	**21,027**

The Kallisto reference index included a total of 29,757 transcripts with a unique Ensembl transcript ID and associated gene ID. A total of 10,269 of these transcripts currently have no meaningful functional annotation or gene name. All RNA-Seq data from this project has been made available to Ensembl.

A principal components analysis (PCA) of the polyA-selected time-course samples was created using R (utilising Kallisto expression estimates). The PCA plot coloured by time points is shown in Fig 5.1A. In Fig 5.1B, each animal is coloured separately. The time-course is cyclical, with the 0hr and 24hr time points being similar to each other, and 4hr and 7hr samples demonstrating the extremes of each individual's response. This circular expression profile over the time course mirrors that which has been seen in the much denser time course of LPS stimulation of human macrophages based upon CAGE tag sequencing (Baillie *et al.*, 2017).

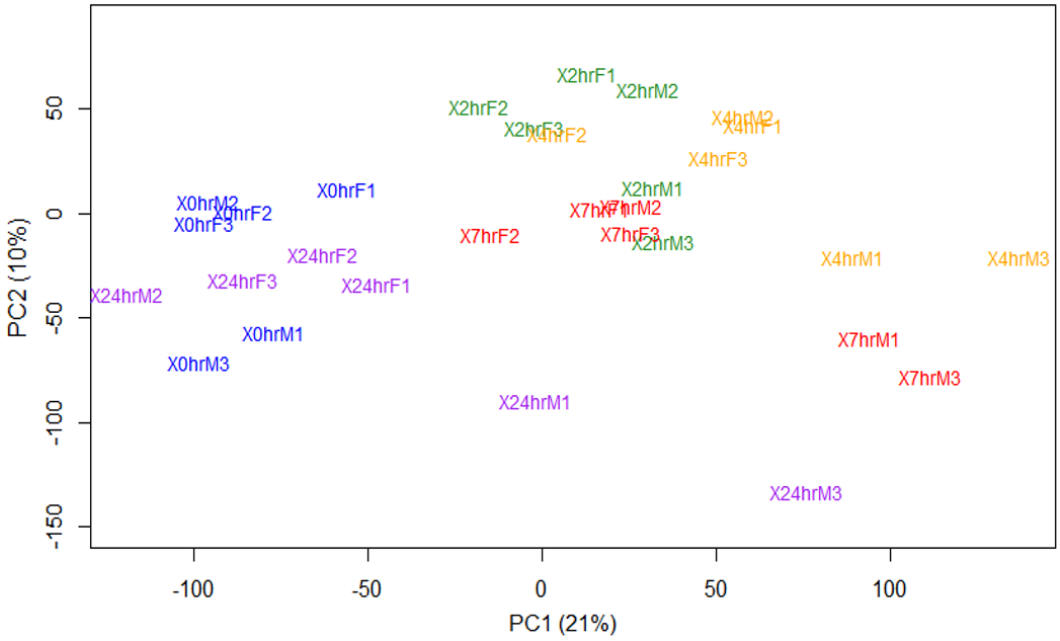
In Figure 5.1B, the three females have all been coloured in shades of pink and red, and the males all in blue. The PCA suggests a possible association of response with sex, however statistically the individual variation is too great and numbers of male and female animals too small to draw any significant conclusions regarding sex influencing the innate response.

Figure 5.1 PCA plot of all polyA-selected BMDM LPS response samples

The biplots were created using Kallisto expression estimates (TPM) for every reference transcript (OarV3.1) across all BMDM response samples from every individual. The % of Variance is shown in brackets for each principal component.

A. PCA plot of BMDM response samples - coloured by time point

0hr as blue, 2hr as green, 4hr as yellow, 7hr as red and 24hr as purple.



B. PCA plot of BMDM response samples - Coloured by animal

Female 1 as red, Female 2 as bright pink, Female 3 as pink, Male 1 as dark blue, Male 2 as light blue and Male 3 as turquoise. Given the number of individuals (3 male and 3 female) and the degree of variation in response, there was not enough power to examine the effect of biological sex on the response.

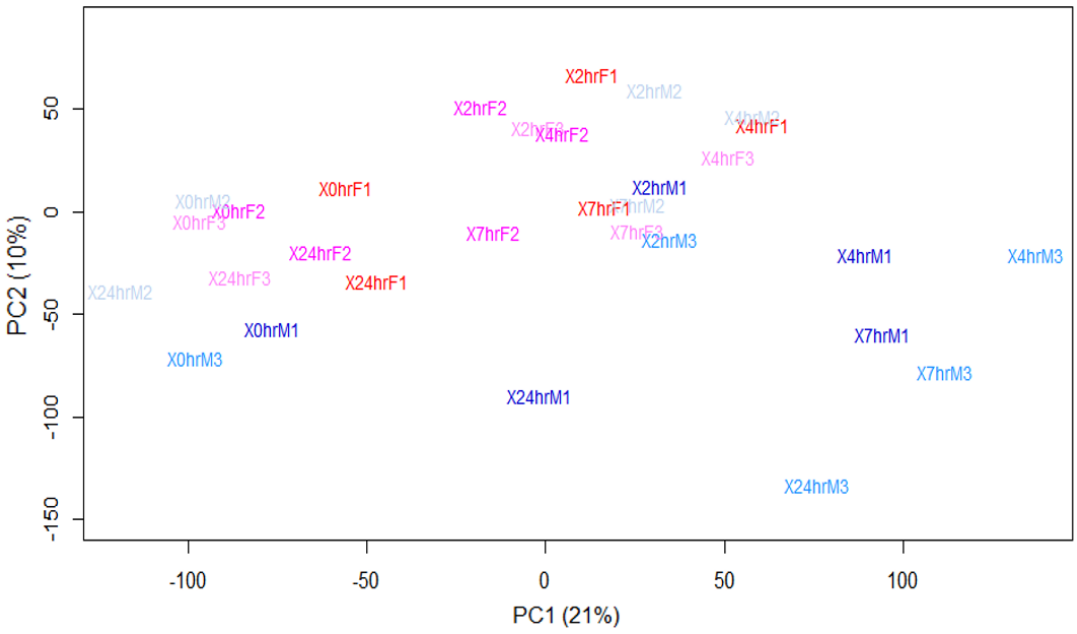


Figure 5.1B shows the significant divergence in the response between individual animals. In every animal, the maximal peak in response to LPS, (the greatest divergence from the 0 hr expression pattern), occurred between 4 and 7 hr. The individual profiles were most similar to each other at zero time, and most divergent at 24 hr. There was a general trend for the 24 hr samples to return towards the unstimulated state. The exception was male 3, which showed no evidence of decay of the response. This phenotype, an apparent lack of feedback control, has been well documented in the control of IL1 production in mice (Brandwein *et al.*, 1987). The response of Female 1, on the other hand, peaked early and rapidly returned to the baseline, suggesting that this sheep is an 'early responder', again a previously documented phenotype in mice (McDermott *et al.*, 2012).

5.3.2 BMDM response clusters from The Sheep Atlas: Clusters 45 and 52

The objective of this section was to extract, analyse and functionally annotate as far as possible the transcripts selectively enriched in LPS-stimulated BMDM from the main Sheep Atlas (Clark *et al.*, 2017). The sheep atlas provides a unique dataset of RNA-Seq data from multiple organ systems across many stages of development ranging from blastocyst to adult sheep from which to extract co-expression information for any given transcript. The clusters enriched in LPS-stimulated BMDM relative to the entire atlas included cluster 45 (containing 44 genes) and cluster 52 (containing 31 genes) (Clark *et al.*, 2017). Where possible, genes possessing only an Ensembl ID (15 genes/75 total genes) have been manually assigned a functional annotation (Appendix 5.1). Every gene identified in these two sheep clusters is also included in later network analyses derived using StringTie or Kallisto (See 5.3.4) or the averaged immune cell analysis (See 5.3.3). The few that do not appear in Section 5.3.4 such as *ENSOARG00000014841* (which has been assigned the functional annotation *CCL1), SOCS3, SLC1A5, TNFSF15* and *PLAUR,* did not show reproducible patterns of regulation in the six individuals and therefore failed to cluster.

GATHER produced identical GO terms for both clusters of genes, including the genes assigned a manual annotation: immune, inflammatory and defence responses; responses to biotic stimulus, wounding, pest pathogen or parasite; external biotic stimulus; stress; communication; taxis; morphogenesis; signal transduction; apoptosis and cell death. These GO terms are consistent with the known biology of the innate immune response.

Many genes included in these clusters are shared with macrophage response clusters in other species, including *IL1A* and *IL1B, IL1RN, SOD2*, multiple TNF superfamily members and

C-C and C-X-C motif chemokines (Freeman *et al.*, 2012, Schroder *et al.*, 2012, Baillie *et al.*, 2017).

Table 5.3 summarises the small number of genes within clusters 45 and 52 that have a distinctive expression profile to those reported in comparable published human, mouse and pig macrophage LPS time course datasets in which cells were cultured with the same sources of CSF1 and LPS (Freeman *et al.*, 2012, Schroder *et al.*, 2012, Baillie *et al.*, 2017).

Table 5.3 Genes identified in the BMDM LPS response clusters (45 and 52) of The Sheep Atlas which have been found to vary in expression pattern across other species.

Ensembl Gene ID and gene name	**Function**	**Expression in other species**
ENSOARG00000006889 SERPINB2 (Assigned)	regulation of protease functions	LPS responsive in all species
ENSOARG00000005159 SERPINB2-like (Assigned)	regulation of protease functions	Duplication shared with cattle (LPS inducible)
ENSOARG00000000835 PNP (Assigned)	catalyses the phosphorolysis of purine nucleosides	Similar response in mice, mild in human, down in pig
ENSOARG00000002754 PTX3	regulation of innate resistance	Similar in human, minimal in mice, not in pig
ENSOARG00000015857 METRNL	Hormone that promotes energy expenditure	Similar in mice, marginal response in human, down in pigs
ENSOARG00000009176 PVR	Transmembrane glycoprotein	No response in human
ENSOARG00000021170 ARG2	catalyses hydrolysis of arginine	Sheep specific
ENSOARG00000010784 SLC1A5	sodium-dependent neutral amino acid transporter	No response in mice, down in humans and pig

Manual annotation of the most differentially expressed genes in cluster 52 revealed a gene duplication of *SerpinB2/PAI-2*, which is shared with cattle (Cattle *SERPINB2: ENSBTAG00000023198/ ENSBTAT00000031553* and Cattle *SERPINB2-like: ENSBTAG00000023026/ ENSBTAT00000031287).* In sheep, *SERPINB2 (ENSOARG00000006889)* had a maximum expression of 9,003 TPM and an expression ratio

of 2477. The second *SERPINB2-like* gene *(ENSOARG00000005159),* also featuring in cluster 52, has two reference transcript models *(ENSOART00000005620* with 7 exons and *ENSOART00000005619* with 8 exons), both of which were increased in response to LPS in all six sheep, with maximum expression estimates of 29 TPM and 2,859 TPM respectively.

Further evidence that this gene duplication was not a genome assembly error was obtained by downloading cDNA sequence data from Ensembl and producing cladogram phylogenetic trees using Clustal Omega (Sievers *et al.*, 2011, McWilliam *et al.*, 2013, Li *et al.*, 2015) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/), to examine all *SERPINB2* genes in a range of different species and all SERPINB genes in both cattle and sheep (not shown).

The greatest homology for the duplicated gene was with *SERPINB2* for both sheep and cattle. *SERPINB2* is profoundly inducible in mouse macrophages (Costelloe *et al.*, 1999) and appears to protect macrophages against apoptosis (Kumar & Baglioni, 1991, Park *et al.*, 2005) and restrict LPS-induced inflammation (de las Casas-Engel *et al.*, 2013, Zhao *et al.*, 2013). Deficiency in *SERPINB2* in mice is linked to impaired CCL2-mediated macrophage influx into the small intestine during infection (Shea-Donohue *et al.*, 2014), and the transcript was upregulated in enteric pathogen infections in both humans and mice (Varro *et al.*, 2004, Schroder *et al.*, 2010, Zhao *et al.*, 2013). The Hume lab has generated RNA-Seq data from cattle BMDM, with and without LPS stimulation (Young *et al.*, 2018). The same expression picture for each *SERPINB2* gene was evident in the cattle data (not shown) suggesting a ruminant specific LPS response adaptation and requirement for a second inhibitor urokinase-type plasminogen activator.

5.3.3 The BMDM response clusters from the immune cell averaged expression network expression graph

To gain an overview of changes in expression across the time course, an initial analysis was performed using values averaged across individuals at each time point (see Section 4.3.2 for methods). A network graph of immune-related cells and tissues including the LPS time course data produced eight macrophage-related and/or LPS-induced clusters as shown in Figure 5.2. These contained a total of 483 reference transcripts. A total of 91 transcripts lacked any functional annotation and were assigned a functional annotation where possible. All details of transcripts contained within each cluster can be found in Appendix 4.1.

Figure 5.2 Identification of LPS responsive BMDM enriched clusters
The clusters containing BMDM response transcripts that increase in expression in response to LPS, were extracted from the network analysis used in chapter 4. The co-expression network layout was based upon average expression across Sheep Atlas immune cell types, including transcripts expressed >30 TPM in any sample. The network contains 6,654 nodes, 1,210,699 edges and was based on a Pearson correlation co-efficient >0.9. Edges (connecting grey lines) represent the correlation between expression patterns of transcripts at a Pearson correlation coefficient of 0.9 or greater. Nodes of the same colour form a cluster. The image on the left shows the whole region; the central image shows the BMDM response clusters in this network. The corresponding signal histograms showing the average expression of transcripts within each cluster are displayed next to the extracted cluster. From left to right, the bars are labelled along the X axis as: BMDM 0hr, 2hr, 4hr, 7hr, 24hr post LPS, AM, BL, MDM, PBMC. All transcripts identified can be examined in Appendix 4.1, LPS responsive worksheet.

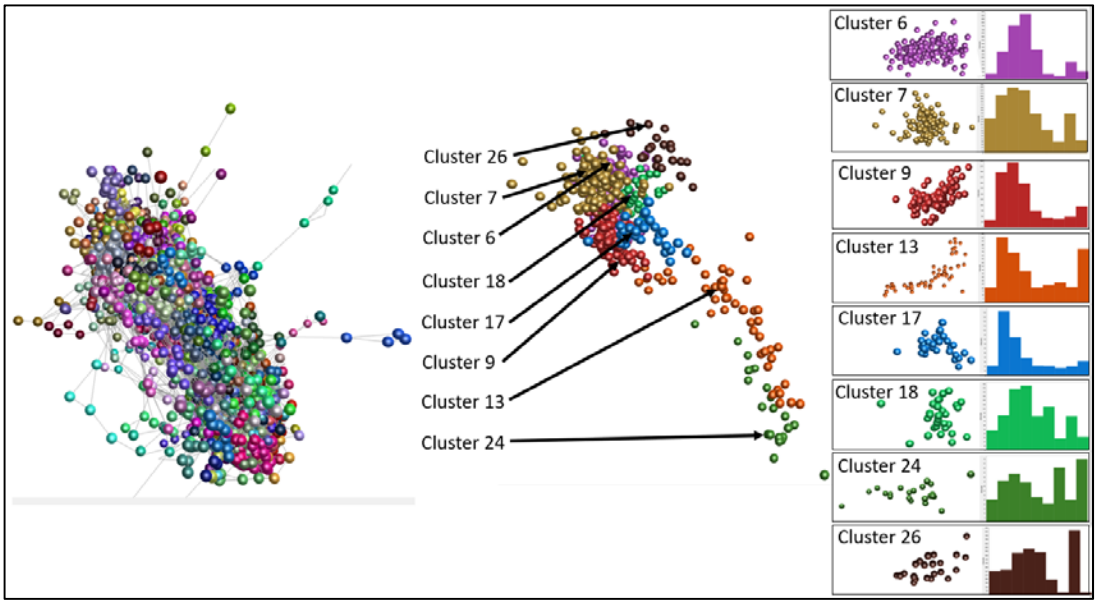


Table 5.4 summarises transcripts identified which appear to show a unique expression pattern in sheep, their inferred function and comparison to the published data on regulation in other species (Freeman *et al.*, 2012, Schroder *et al.*, 2012, Baillie *et al.*, 2017). All references to comparative expression in the text below refer to the same datasets unless otherwise indicated.

Table 5.4 Sheep-specific LPS responsive genes

Transcripts and their corresponding gene ID and names were identified from the averaged immune cell analysis, and found to differ absolutely in expression pattern in comparison to other species (Freeman *et al.*, 2012, Schroder *et al.*, 2012, Baillie *et al.*, 2017). The function assigned to each gene is based upon information from www.genecards.org.

Ensembl IDs and gene name	**Function of encoded protein**	**Expression in other species**
ENSOART00000008204 ENSOARG00000007533 ADCYAP1R1	Type I adenylate cyclase activating polypeptide receptor, mediated by G proteins. Activating adenylate cyclase involved in transmembrane signalling and shares homology with members of the glucagon/secretin receptor family.	Sheep specific Predominantly CNS and not responsive to LPS in human, mouse or pig.
ENSOART00000017157 ENSOARG00000015761 HTR7	5-Hydroxytryptamine Receptor 7. Receptor for 5-hydroxytryptamine (serotonin), a biogenic hormone that functions as a neurotransmitter, a hormone, and a mitogen. Mediated by G proteins that stimulate adenylate cyclase.	Response shared with pig, less so in mouse, no response to LPS in human
ENSOART00000023097 ENSOART00000023098 ENSOARG00000021196 TTC9 (assigned)	Tetratricopeptide Repeat Domain 9	Sheep specific
ENSOART00000016356 ENSOARG00000015024 PRKAR1A	Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha. Activates the cAMP-dependent protein kinase, which transduces the signal through phosphorylation of different target proteins.	Sheep specific
ENSOART00000004064 ENSOART00000004065 ENSOARG00000003740 SUCNR1	Succinate Receptor 1. G-protein-coupled receptor for succinate, an intermediate molecule of the citric acid cycle.	Sheep specific
ENSOART00000009357 ENSOARG00000008597 SGMS1	Sphingomyelin Synthase 1. Five-pass transmembrane protein. Synthesise the sphingolipid, sphingomyelin, through transfer of the phosphatidyl head group, phosphatidylcholine, on to the primary hydroxyl of ceramide. The reaction is bidirectional depending on the respective levels of the sphingolipid and ceramide.	Responsive in sheep, pig and mouse, not in human
ENSOART00000009357 ENSOARG00000008597 SGMS2	Sphingomyelin Synthase 2. Enzyme that catalyses production of Sphingomyelin (which affects insulin sensitivity), a sphingolipid and major component of cell and Golgi membranes, required for cell growth. Regulates cell surface levels of ceramide, mediating signal transduction and apoptosis.	Sheep specific

Ensembl IDs and gene name	Function of encoded protein	Expression in other species
ENSOART00000017366 ENSOARG00000015947 GLRX5	Glutaredoxin 5, Mitochondrial protein, involved in the biogenesis of iron-sulphur clusters, required for iron homeostasis. Involved in protein lipoylation. Required for normal regulation of haemoglobin synthesis by the iron-sulphur protein ACO1.	Decreased in response to LPS in mouse, human and pig
ENSOART00000000937 ENSOARG00000000875 TPM4	Tropomyosin 4. Actin-binding protein involved in the calcium dependent regulation of contractile system of striated and smooth muscles and stabilising the cytoskeleton of non-muscle cells. Binds calcium.	Highly LPS responsive in mouse. Decreased in response to LPS in human
ENSOART00000012596 ENSOARG00000011582 CYP26B1	Cytochrome P450 Family 26 Subfamily B Member 1. Monooxygenase which catalyse many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. Regulator of all-trans retinoic acid levels by the specific inactivation of all-trans retinoic acid to hydroxylated forms.	Sheep specific
ENSOART00000010021 ENSOARG00000009205 CDA	Cytidine Deaminase. Enzyme which scavenges exogenous and endogenous cytidine and 2-deoxycytidine for UMP synthesis, may also participate in RNA editing.	Decreased in response to LPS in human
ENSOART00000006066 ENSOARG00000005565 ADSS	Adenylosuccinate Synthase. Enzyme which catalyses conversion of inosine monophosphate to adenosine monophosphate.	Decreased in response to LPS in mouse, human and pig
ENSOART00000006753 ENSOARG00000006212 CRABP2	Cellular Retinoic Acid Binding Protein 2. Member of the retinoic acid binding protein family and lipocalin/cytosolic fatty-acid binding protein family.	Marginal response in pig, absent in mouse. Decreased in response to LPS in human
ENSOART00000006301 ENSOARG00000005787 WFS1	Wolframin ER Transmembrane Glycoprotein. Partly regulates cellular Ca^{2+} homeostasis, by modulating the filling state of the endoplasmic reticulum Ca^{2+} store.	Sheep specific
ENSOART00000002003 ENSOARG00000001863 SEMA6A	Semaphorin 6A. Expressed in developing neural tissue and required for proper development of the thalamocortical projection, promoting reorganization of the actin cytoskeleton and important axon guidance cue.	Sheep specific
ENSOART00000006465 ENSOARG00000005941 TNC	Tenascin C. Extracellular matrix protein, containing multiple EGF-like and fibronectin type-III domains.	Induced by LPS in human, unresponsive in pig and mouse

Ensembl IDs and gene name	Function of encoded protein	Expression in other species
ENSOART00000019500 ENSOARG00000017919 SHTN1	Shootin 1. Important role in regenerative neurite outgrowth and the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3).	Downregulated in mouse and pig LPS response, no response in humans
ENSOART00000018427 ENSOARG00000016918 F2RL2(PAR3)	Coagulation factor II thrombin receptor like 2. G-protein-coupled receptor that encodes protease-activated receptor-3.	Sheep specific
ENSOART00000011926 ENSOARG00000010953 XDH	Xanthine Dehydrogenase. Molybdenum-containing hydroxylase involved in purine degradation, regulating adipogenesis and peroxisome proliferator-activated receptor-γ (PPAR-γ)	Sheep specific
ENSOART00000000285 ENSOARG00000000271 CLMP (ACAM)	CXADR like membrane protein. Type I transmembrane protein and involved in cell-cell adhesion	Sheep specific

All time course samples were PolyA-selected for the RNA-Seq library prep method (see chapter 2.7) which should remove most non-coding transcripts. Of the 15 transcripts that were not protein-coding, yet apparently highly expressed and regulated in response to LPS, 8 are lincRNAs, 7 are miRNAs, 4 are pseudogenes, 2 are rRNAs, 1 is a snoRNA and 4 were snRNAs, suggesting that these transcripts belonging to other classes of RNA are functionally important to the sheep in responding to LPS.

From these selected BMDM LPS responsive enriched clusters from the immune cell network expression graph, 73 reference transcripts lacked any functional name. All have been examined in Ensembl, their nearest orthologues and syntenic regions compared and they have been assigned a 'Preliminary annotation' (see Appendix 4.1, LPS responsive worksheet).

Clusters 13 and 17 contain transcripts that were induced transiently, peaking after 2 hours. The basal level of expression is broadly shared by all other immune cells in cluster 17. However, in cluster 13, the transcripts are also enriched in the PBMCs (Figure 5.2). Transcripts encoding inflammatory cytokines such as TNF, with associated signal transduction molecule TRAF1, along with genes for surface receptors such as CD83, early transcription factors such as FOSL1 and feed forward activators such as TRAF1, TICAM1, RCAN1, IRAK2 all featured in this first 'wave' of increased transcription. The pattern of

expression for these transcripts is largely shared with mouse and human (Nilsson *et al.*, 2006, Baillie *et al.*, 2017). Amongst the highest expressed transcripts was *ENSOART00000012146* annotated as *CEBPD*, a gene encoding a transcriptional activator, and *ENSOARG00000009537* annotated as *DUSP5*. Transcripts with the highest expression ratio in cluster 13 include a novel miRNA (*ENSOARG00000024480*) and *NR4A3*, which encodes a transcriptional activator and member of the steroid-thyroid hormone-retinoid receptor superfamily.

Clusters 7, 9 and 24 contain transcripts that also increased transiently, peaking at 4 hr in response to LPS. Cluster 9 is specific to the BMDM response. Cluster 7 shows some enrichment in the MDMs, possibly reflecting the similar culture methods as MDM and BMDM are cultured in the presence of rhCSF1. Cluster 24 shows some enrichment in BLs and PBMCs as well as increasing in response to LPS in the BMDM samples.

Cluster 9 contains multiple chemokines and cytokines including IL6. Some were previously unannotated: *ENSOARG00000025179* annotated as *CXCL1, ENSOARG00000014841* annotated as *CXCL2*. The cluster also contains genes for multiple signalling molecules, including the tyrosine kinase JAK2 and downstream kinase MAP2K3, transcription factors such as FOSL2 and nuclear hormone receptor Vitamin D receptor (VDR) and the receptors TLR2 and TLR4. *GPR84* (encoding a G protein-coupled receptor 84, detecting C9-C14 medium chain free fatty acids) is also found in cluster 9, sharing the same dramatic increase in expression seen in humans, mice and pigs.

Cluster 24 contains genes for the key enzyme in prostaglandin biosynthesis, cyclooxygenase 2 (PTGS2) (Kawahara *et al.*, 2015, Tang *et al.*, 2017) and NIPAL1, a transmembrane transporter of divalent cations (Goytain *et al.*, 2008) alongside known acute response genes that are associated with TRAF signalling like *BIRC3, TRAF2* and *TANK* (Hacker *et al.*, 2011).

Clusters 6, 18 and 26, containing transcripts that peak at 7hrs.

The largest, cluster 6 includes further inflammatory cytokine genes such as the colony stimulating factors *CSF1, CSF2* and *CSF3,* Interleukins *IL1A* and *IL1B* and many C-C and C-X-C motif chemokines that are also amongst the most highly-induced transcripts in all other species examined. Inflammatory resolving transcripts include both transcript models for the cytokine-inducible negative regulator of cytokine signalling, *CISH*. Genes encoding less

well-known mediators that are nevertheless shared with other species include *EREG* and *INHBA* and transcription factors *ETS2* and *AEBP2* (assigned to *ENSOARG00000020470)*.

Many of the 30 transcripts contained in cluster 18 centre around transcriptional control, growth, adhesion and cytoskeleton organisation and endocytic trafficking, such as *ATF6, PPARD, SHTN1* and the highly inducible novel miRNA *ENSOART00000025421/ ENSOARG00000021041*.

Cluster 26 contains *ENSOARG00000005159*, one of the most differentially expressed transcripts which has been assigned the functional annotation of *SERPINB2* (see Section 5.3.2). *CCL2* (encoding a monocyte-attracting chemokine) is the most highly expressed transcript in this cluster, a chemokine that mediates monocyte recruitment, enhancing LPS production of IL-10, with high expression indicative of macrophages under the influence of CSF1 (Sierra-Filardi *et al.*, 2014). Another very highly expressed transcript, *ENSOART00000010843/ ENSOARG00000009963,* was annotated as a serum amyloid gene, *SAA3,* encoding a well-known acute phase protein (Zhou *et al.*, 2017) and sharing an expression pattern with other species. *ADGRE1*, which encodes the widely used macrophage marker, F4/80 in mice (Austyn & Gordon, 1981, Hume *et al.*, 1984) was also highly expressed and further LPS inducible in sheep.

Analysis of the resolution phase of the response at 24 hr necessitates separate examination of the individual response signatures (Section 5.3.9), because of the divergent nature of the response between individuals (Section 5.3.1). There were no shared clusters found to peak in expression in the resolution phase (24 hrs).

5.3.4 Network analysis of the BMDM response to LPS in individual sheep.

The initial analyses (Section 5.3.3) used averaged values. However, it was clear from the PCA (Section 5.3.1) that some averages would be highly skewed by the expression level in a single individual. Therefore, the macrophage LPS response was assayed in isolation capturing all regulated transcripts regardless of their restriction to macrophages. The Kallisto sample-sample network in Fig 5.3 (Appendix 5.7 and 5.9) clearly mirrors the PCA plots in Fig 5.1, in comparison to StringTie, which highlights the extreme individual response of Male 3.

Figure 5.3 Sample-based networks comparing StringTie and Kallisto expression estimates from individual sheep BMDM time course samples.
For StringTie, all genes which demonstrated maximum expression > 5 FPKM in any sample across the time course were included. For Kallisto, all transcripts which demonstrated a maximum expression >1 TPM in any sample across the time course were included. All genes and transcripts for both pipelines had an expression ratio ((Maximum+1)/(Minimum+1)-1) > 1.5. The Pearson correlation coefficient threshold was 0.92. Each node represents a sample and the lines between them represent correlations of at least 0.92. Nodes are coloured according to the sample time point.

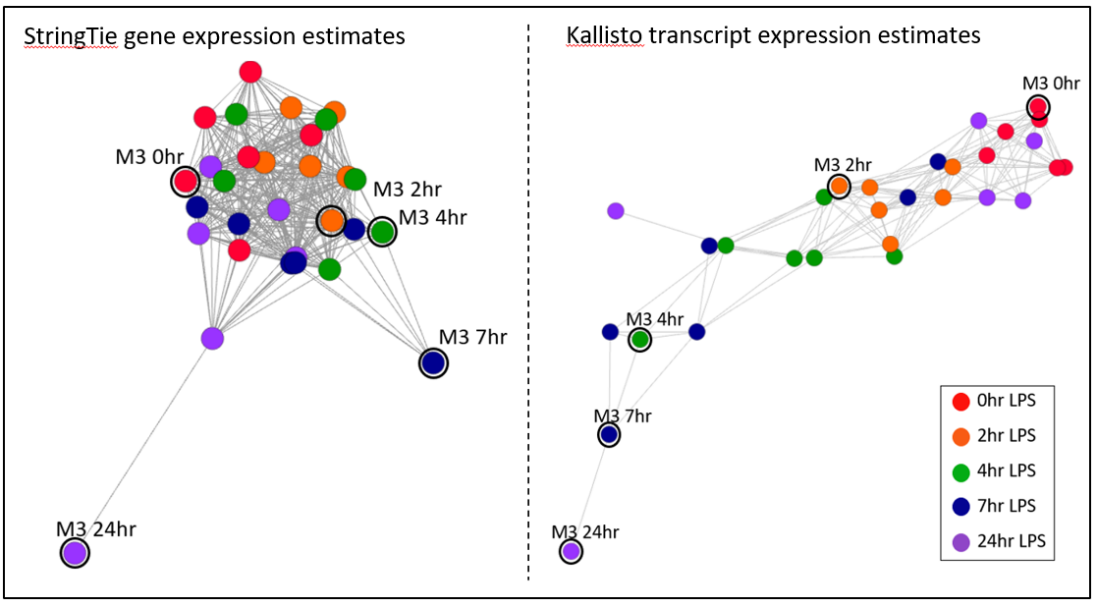
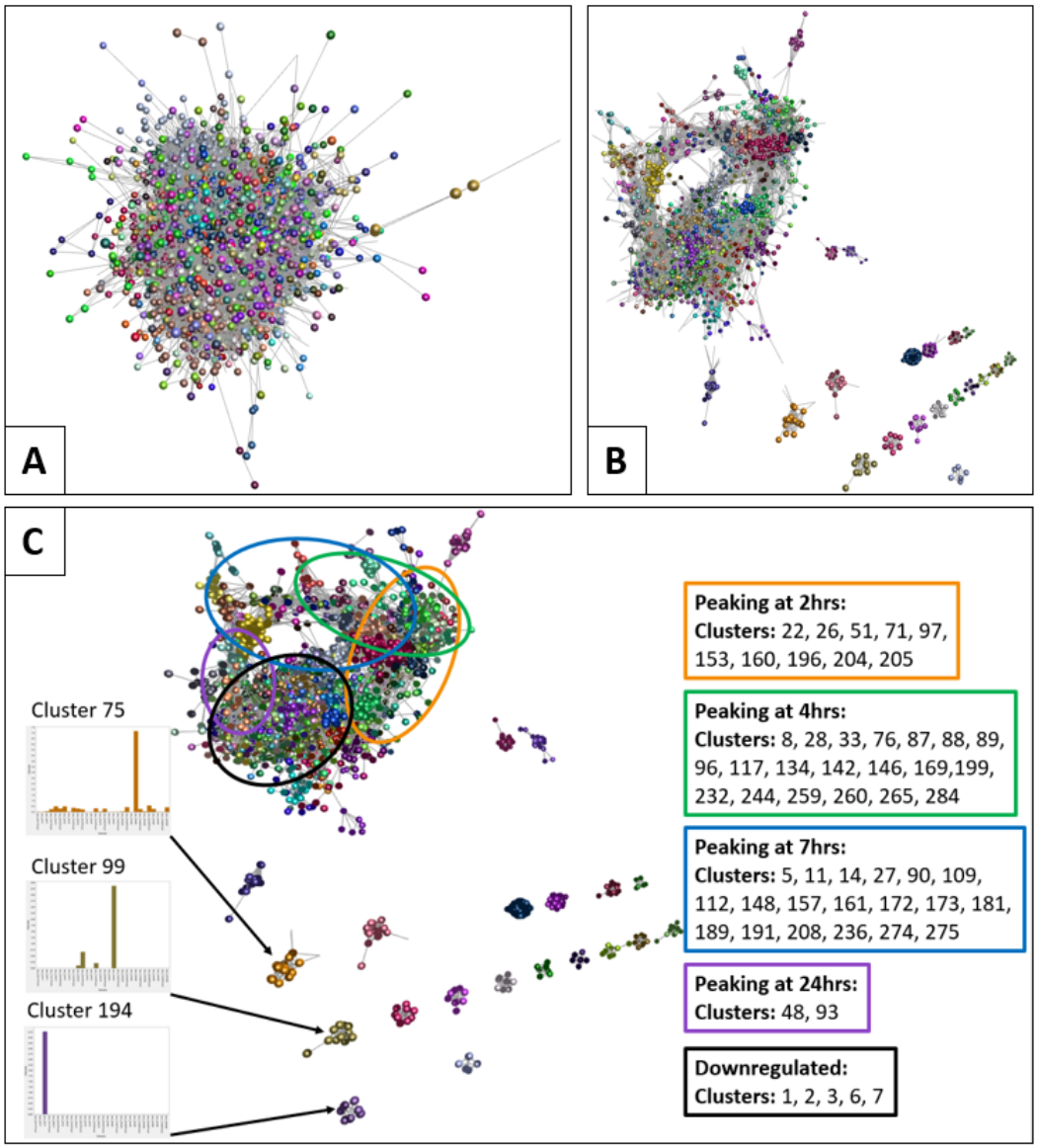


Figure 5.4 shows the gene-based networks for both methods, using the values for individual sheep. The network expression graph using StringTie expression estimates (Figure 5.4 A), is more tightly-clustered than that based on Kallisto expression estimates (Figure 5.4 B) where the circular temporal response is clearly evident (Figure 5.4 C). The smaller discrete clusters, highlighted by clusters 75, 99 and 194, contain miRNAs and snoRNAs specific to certain individuals and were not found to contain highly expressed protein coding genes.

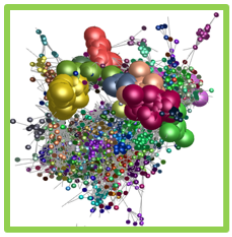
Figure 5.4 BMDM response gene-based network expression graphs
A. Network expression graph using StringTie expression estimates for every BMDM response sample from each individual sheep (see Section 5.2.2 for methods)
B. Network expression graph using Kallisto expression estimates for every BMDM response sample from each individual sheep (See Section 5.2.1 for methods)
C. The Kallisto-based network highlighting clusters of genes that appear regulated during the response to LPS.



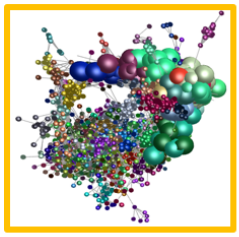
Figures 5.4 C and 5.5 emphasise the circular nature of this temporal response, in common with human macrophages (Baillie *et al.*, 2017), and Figure 5.5 highlights exactly where each cluster of interest is to be found within the main network expression graph and the associated GO terms (Chang & Nevins, 2006).

Figure 5.5 Kallisto expression estimate network graph highlighting each key cluster of interest.

Nodes in the relevant clusters for that 'peaking time point' are enlarged in each panel for clarity, to show their position within the overall network graph. Transcripts contained within each cluster can be viewed along with the associated signal histogram for each cluster in the relevant 'peaking' spreadsheet within Kallisto Peaking Analysis Excel workbook in Appendix 5.2. The GO terms across all clusters peaking at a given time point are derived using GATHER (Chang & Nevins, 2006)



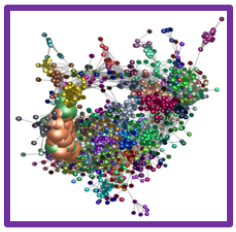
Clusters Peaking at 7hrs:
5, 11, 14, 27, 90, 109, 112, 148, 157, 161, 172, 173, 181, 189, 191, 208, 236, 274, 275



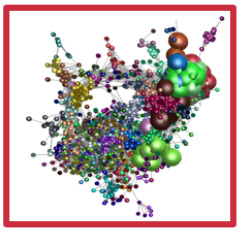
Clusters Peaking at 4hrs:
8, 28, 33, 76, 87, 88, 89, 96, 117, 134, 142, 146, 169,199, 232, 244, 259, 260, 265, 284

Peak response: 4hrs, 7hrs
angiogenesis
blood vessel development
cell migration
cell motility
cell poliferation
cellular morphogenesis
cellular physiological process
embryonic development
embryonic morphogenesis
intracellular signalling cascade
perception of smell
posiive regulation of metabolism
regulation of cell cycle
regulation of cell proliferation

Peak response:2hrs, 4hrs and 7hrs
apoptosis
cell death
cell differentiation
death
detection of external stimulus
G-protein coupled receptor protein signalling
humoral immune response
positive regulation of cellular physiology
positive regulation of physiological processes
programmed cell death
regulation of apoptosis
regulation of biological process
regulation of cellular physiological processes
regulation of organismal physiological processes
regulation of programmed cell death
sensory perception



Clusters Peaking at 24hrs:
48, 93



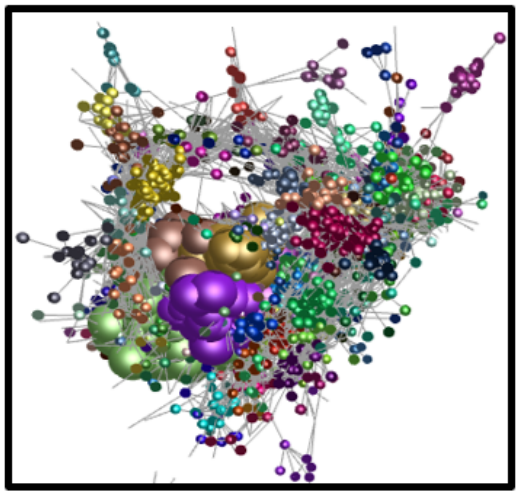
Clusters Peaking at 2hrs:
22, 26, 51, 71, 97, 153, 160, 196, 204, 205

Resolution: 24 hrs
alcohol catabolism
alcohol metabiolism
carbohydrate catabolism
cell activation
cell surface receptor linked signal trasduction
cellular carbohydrate catabolism
cellular carbohydrate metabolism
energy derivation by oxidation of organic compounds
generation of precursor metabolites and e...
glucose catabolism
glucose metabolism
glycolysis
Hexose catabolism
hexose metabolism
immune cell activation
lymphocyte activation
main pathways of carbohydrate metabolism
monocarboxylic acid transport
monosaccharide catabolism
monosaccharide metabolism
muscle contraction
nucleobase, nucleoside, nucleotide and nucleotide kinase activity
Response to abiotic substance

Early response: 2hrs, 4hrs
cellular defense response
protein folding
protein kinase cascade
response to heat
response to temperature
neurophysiological process
phosphate metabolism
phosphorus metabolism
phosphorylation
protein amino acid phosphorylation
sensory perception of chemical stimulus

Figure 5.5 Continued.

Downregulated clusters: 1, 2, 3, 6, 7



Downregulated in response
biological process unknown
Cell cycle
cell organization and biogenesis
cellular macromolecule metabolism
cellular metabolism
cellular process
cellular protein metabolism
DNA metabolism
DNA repair
DNA replication
DNA replication and chromosome cycle
DNA-dependent DNA replication
macromolecule metabolism
metabolism
organelle organization and biogenesis
physiological process
primary metabolism
protein metabolism
protein modification
response to DNA damage stimulus
response to endogenous stimulus

Shared: down and increased response
cell communication
cell poliferation
cell surface receptor linked signal transduction
cellular morphogenesis
cellular physiological process
detection of external stimulus
development
embryonic development
G-protein coupled receptor protein signalling
intracellular signalling cascade
morphogenesis
neurophysiological process
organ development
organismal physiological process
organogenesis
perception of smell
phosphate metabolism
phosphorus metabolism
phosphorylation
protein amino acid phosphorylation
regulation of biological process
regulation of cell cycle
regulation of cellular physiological processes
regulation of cellular process
regulation of physiological process
response to external stimulus
response to stress
sensory perception
sensory perception of chemical stimulus
signal transduction

Shared: peaking 2hrs, 4hrs, 7hrs and 24hrs
cell communication
cell-cell signaling
chemotaxis
defense response
development
humoral defense mechanism (sensu Vertebrates)
immune response
inflammatory response
morphogenesis
organ development
organismal physiological process
organogenesis
regulation of cellular process
response to biotic stimulus
response to chemical substance
response to external biotic stimulus
response to external stimulus
response to pest, pathogen or parasite
response to stimulus
response to stress
response to wounding
signal transduction
taxis

5.3.5 Down-regulated transcripts in the response to LPS

In common with the other similar projects in human, mouse and pig, when individual values were analysed, the largest clusters (1-3) contain transcripts that decrease during the response to LPS (as shown in Figure 5.6), reaching a nadir around 7 hours. Many of the genes within clusters 1 and 2 were similarly repressed in response to LPS in mouse, human and pig macrophages. In Female 1 (the early responder) expression returned to pre-stimulation levels at 24 hours, whereas the same transcripts remained down-regulated in Male 3.

Clusters 1 and 2 were enriched for GO terms; sensory perception of chemical stimulus; cell proliferation; cell cycle; detection of external stimulus; G protein coupled receptor protein signalling; cellular physiological process; neurophysiological process; protein amino acid phosphorylation; response to stress; primary metabolism; protein modification.

This pattern reflects the known ability of LPS to block cell proliferation in macrophages, in part by down-regulating the CSF1 receptor (Sester *et al.*, 1999, Sester *et al.*, 2005). The most highly downregulated transcript was *adenosine A3 receptor (ADORA3)*, a gene that is not expressed robustly in either mouse or human macrophages and in the pig appears to increase in response to LPS. This gene encodes a G-protein coupled receptor, that has been implicated in mediating both cell proliferation and death (Salvatore *et al.*, 2000).

Two transcripts encoding uncharacterized proteins *(ENSOART00000020222/ ENSOARG00000018581* and *ENSOART00000020267/ ENSOARG00000018624)* that are both members (the only members) of the same protein family PTHR24365_SF250, with Toll/interleukin-1 receptor homology domains, were also highly-expressed in unstimulated cells and rapidly down-regulated. Their function is unknown.

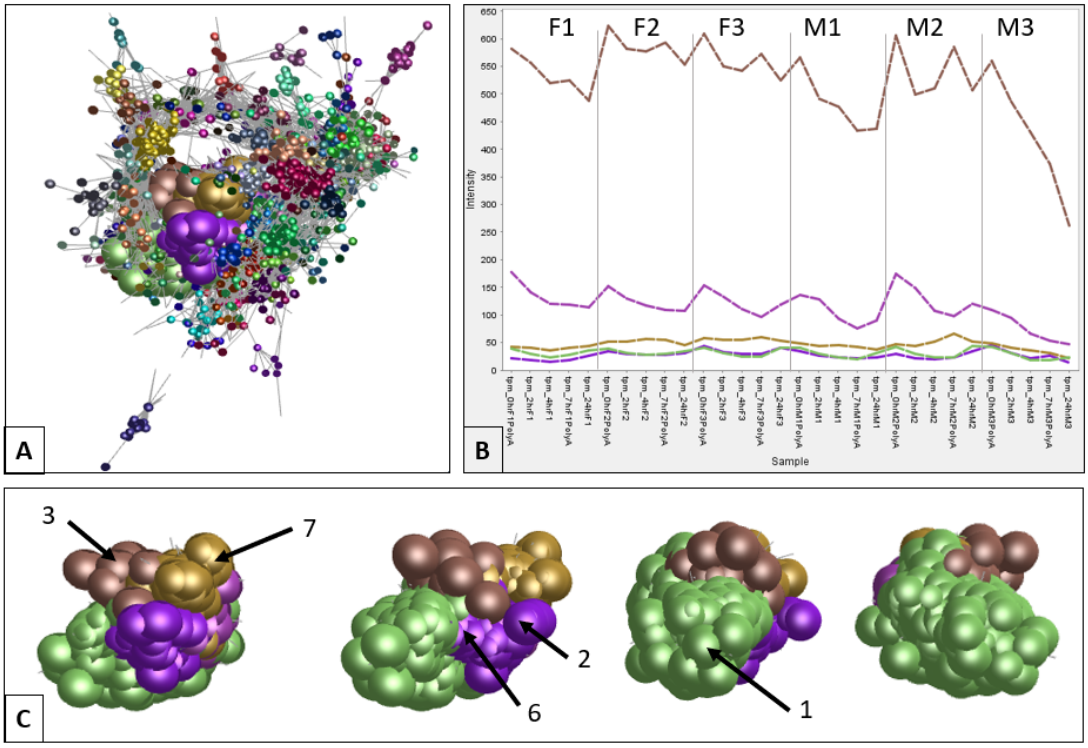
In cluster 2 one of the most down-regulated transcripts was *ENSOART00000005567/ ENSOARG00000005113* assigned to *FAM72A*, a gene implicated in the regulation of ROS metabolism and cell growth (Schieber & Chandel, 2014, Zhao *et al.*, 2017). In mouse BMDM, *FAM72A* is also highly-expressed, transiently induced but then repressed by LPS (BioGPS.org). In humans, it does not appear to be expressed in myeloid cells.

Figure 5.6 Identification of clusters with decreased expression over time in response to LPS

A Location of the key downregulated clusters (enlarged nodes) in the wider network expression graph.

B Mean expression of transcripts contained in each cluster across individuals: Female 1 (F1), Female 2 (F2), Female 3 (F3), Male 1 (M1), Male 2 (M2) and Male 3 (M3) and across time points 0hr, 2hr, 4hr, 7hr and 24 hr (left to right for each individual). Each line represents the cluster of the same colour

C The collection of downregulated clusters. Successive images rotated by 45 degrees.



5.3.6 Inter-individual variation in the early response phase

Clusters which peak in expression at 2hrs after LPS stimulation in most of the individuals have been extracted from the network expression graphs (Figure 5.7) created using values for individual sheep with both Kallisto (Section 5.2.1) and StringTie (Section 5.2.2) and can be viewed in Appendix 5.2 and Appendix 5.3 respectively. Many genes have been assigned a functional annotation, including genes encoding immediate early transcription factors that share similar expression profiles with humans, mice and pigs, *EGR1* (assigned to *ENSOART00000017938/ ENSOARG00000016470* in cluster 51) and *EGR4* (assigned to *ENSOART00000012890/ ENSOARG00000011851* in cluster 153). Other inducible transcripts shared with humans and mice include genes encoding AP1 transcription factor subunits, *FOSB, FOSL1* and *FOSL2* and *MAP3K2,* encoding a signalling kinase, in cluster 26.

Fig 5.7 A illustrates the positioning of these clusters within the wider network analysis described in Section 5.2.1, showing the close proximity to one another, with cluster 196 (where female 2 shows the greatest expression at 2hrs) more disparate from others such as cluster 97 (Fig 5.2C). Cluster 97 strongly demonstrates inter-individual variation, with female 1 showing particularly high expression for all the transcripts within the cluster. *HSPA5* and *IL23A* are highly expressed and appear to be co-regulated in this cluster. Future work could investigate possible shared transcription factor binding sites for these genes.

Cluster 97 is mirrored in cluster 13 of the StringTie analysis, which contains many of the same genes, including *IL23, HSPA5, MCM3Ap, TCEAL1, NRD1* and *SLC25A30* (assigned to *ENSOARG00000007620/ ENSOART00000008296*). A total of 126 genes are contained in StringTie cluster 13, which include 25 novel gene models that are not associated with an Ensembl ID. Many of these are likely spurious models such as *MSTRG.4791* which is only 273 base pairs in length and only contains one exon. Conversely, some novel models are more robust, such as *MSTRG.3436* which has 3 exons and is 2988 base pairs in length.

Figure 5.7 Visualisation of the clusters demonstrating a regulated peak in response to LPS at 2hrs

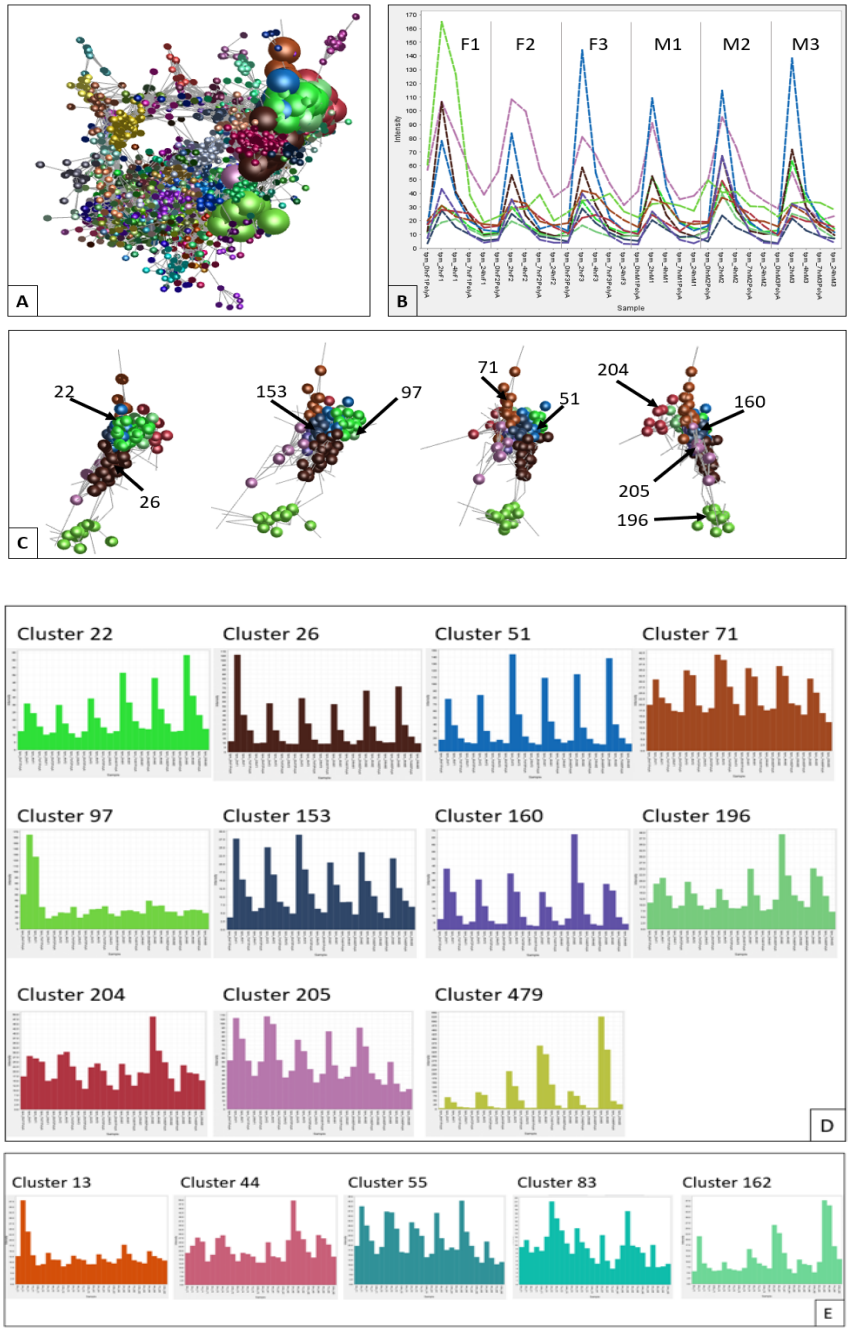
A. Key clusters found to peak in expression at 2hrs (enlarged nodes) in the wider network expression graph using Kallisto in 5.2.1.

B. Mean expression of transcripts contained in each cluster of 5.2.1 across individuals: Female 1 (F1), Female 2 (F2), Female 3 (F3), Male 1 (M1), Male 2 (M2) and Male 3 (M3) and across time points 0hr, 2hr, 4hr, 7hr and 24 hr (left to right).

C. Clockwise rotation of the collection of clusters peaking at 2hrs.

D. Signal histogram associated with each cluster peaking at 2hrs, using Kallisto (5.2.1) expression estimates

E. Shows the signal histogram associated for each cluster found peaking at 2hrs, using StringTie (5.2.2) expression estimates, with labels for the x axis, as listed in B.



Clusters 22 and 479 of the Kallisto network are particularly enriched with transcripts peaking at 2 hr that were very highly expressed in Male 3. Included in these clusters are transcripts associated with acute phase inflammation such as *TNF, NLRP3* (both in cluster 22) and *TRIM, TNFSF9, MAPKAPK2* alongside genes encoding important transcription factors such as *TCF7, KLF2* (assigned to *ENSOARG00000019122/ ENSOART00000020822*) and *FOSL1*.

The equivalent StringTie cluster, containing transcripts enriched for immediate response genes with the greatest expression in Male 3 above all other individuals is cluster 162. *NFKBIZ* is identified in this cluster, which is contrary to the Kallisto network analysis that identifies the only reference transcript for this gene (with 11 exons) within cluster 8 to peak at 4hrs. On closer inspection, StringTie has identified 5 additional transcript models for this highly-expressed and regulated transcriptional regulator (not shown). In humans there are 11 splice variants for *NFKBIZ*, illustrating how this RNA-Seq data having been made available to Ensembl will help improve annotation, coverage and recognition of more transcript variants in the future updated version of the sheep genome OarV5.

Figure 5.8 Expressed *NFKBIZ* transcripts across the time course
StringTie expression estimates (FPKM) of the various transcript variants of NFKBIZ across the time course for each individual animal.

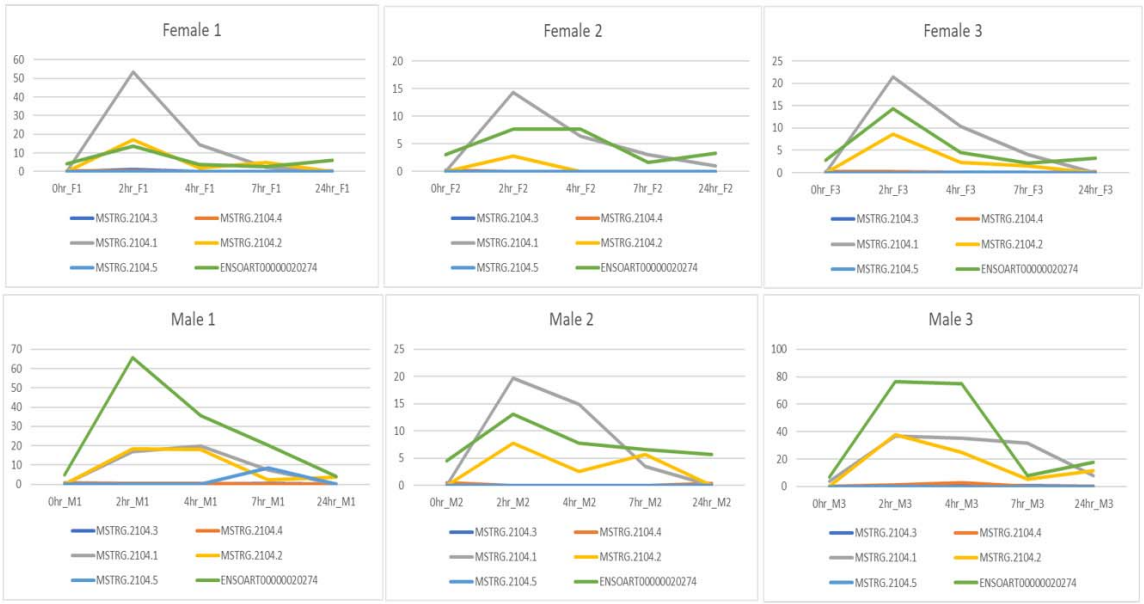
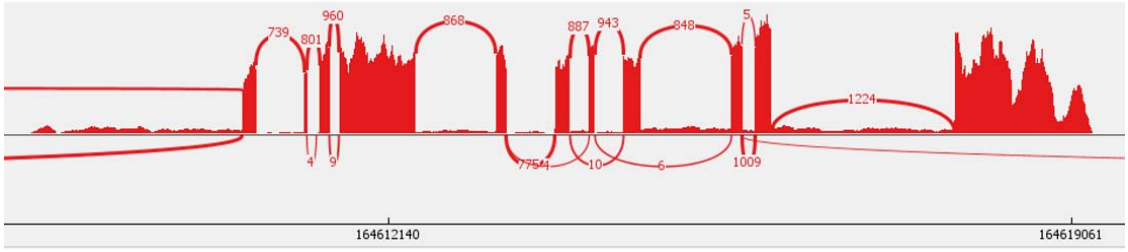


Figure 5.8 continued: Expressed *NFKBIZ* transcripts across the time course
B. Sashimi plot of *NFKBIZ* for 2hr LPS response sample from Male 3.

.



5.3.7 Inter-individual variation at the mid early response phase

Clusters which peaked in expression at 4 hr after LPS stimulation in most of the individuals were extracted from the network expression graphs (Figure 5.9) created using both Kallisto (5.2.1) and StringTie (5.2.2) with individual values and can be viewed in Appendix 5.2 and Appendix 5.3 respectively.

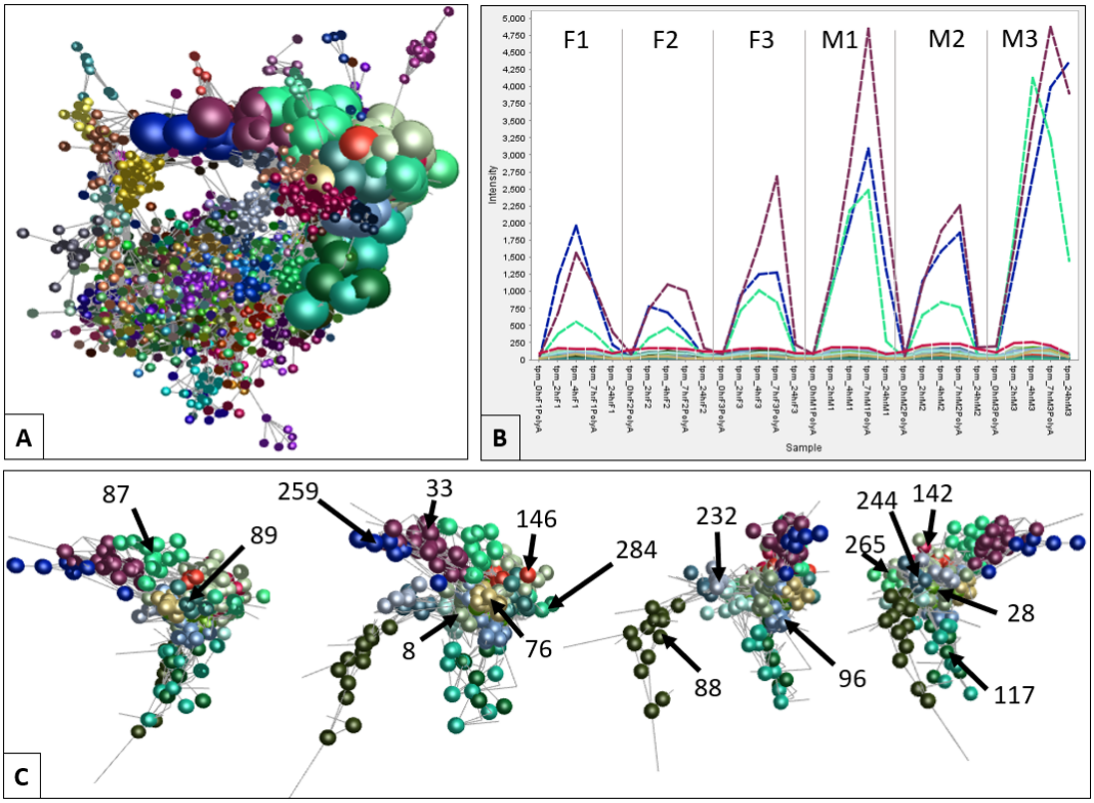
For both pipelines, out of all the clusters found to peak in expression at 4 hr, the most highly-expressed and regulated gene was the acute phase inflammatory cytokine *IL1B (Ren & Torres, 2009)*. Kallisto assigned expression to a single transcript model: *ENSOART00000022733* (6 exons), whereas StringTie assigned expression estimates to two transcript models: the reference model (6 exons, Chr3: 60023514-60034494) and *MSTRG.21999.1* (8 exons, Chr3: 60022671-60048960). Based upon the StringTie analysis, only males 1 and 2 demonstrate robust, regulated expression of the reference transcript model, peaking at 7 hr (not shown). This suggests a differentially regulated splice variant of *IL1B* exists in some individuals.

Figure 5.9 shows the clusters peaking at 4hrs within the main Kallisto network analysis in greater detail. Clusters 33, 87 and 259 contain the most highly regulated and expressed transcripts encoding many inflammatory cytokines such as *IL1B, IL1A, IL11* and colony stimulating factors *CSF1, CSF2* and *CSF3* and multiple transcripts encoding C-X-C motif chemokines. In each case, male 3 showed the highest expression level.

Male 3 also had the highest expression of the majority of transcripts in cluster 6 from the StringTie network. In addition to those identified in the Kallisto-based network, cluster 6 also contains *IL6* and downstream interferon response and signalling genes such as *IRF3* and *IRF7, IFIT2* and *IFIT5, CCL4, CCL5, CCL8* and *CCL20, RIPK1* and *STAT2*. There are 336 genes in this interferon response dominated cluster and 57 are novel genes not associated

with any Ensembl ID. Two of these novel transcripts *MSTRG.9285* and *MSTRG.5463* were robustly expressed (maximum expression estimates 54 and 34), significantly regulated (Ballgown q values 0.000645 and 0.000000000000177 respectively), relatively long (558 bp and 6117 bp) and encoded by multiple exons.

Figure 5.9 Visualisation of the clusters demonstrating a regulated peak in response to LPS at 4hrs
A. Key clusters found to peak in expression at 4hr in the wider network expression graph.
B. Mean expression of transcripts contained in each cluster across individuals: Female 1 (F1), Female 2 (F2), Female 3 (F3), Male 1 (M1), Male 2 (M2) and Male 3 (M3) and across time points 0hr, 2hr, 4hr, 7hr and 24 hr (left to right).
C. Clockwise rotation by 45 degrees, of the clusters peaking at 4hr.



5.3.8 Inter-individual variation at the peak inflammatory response phase

Clusters which peak in expression at 7 hr after LPS stimulation in most of the individuals were extracted from the network expression graphs (Figure 5.10) created using both Kallisto (5.2.1) and StringTie (5.2.2) with individual values and can be viewed in Appendix 5.2 and Appendix 5.3 respectively.

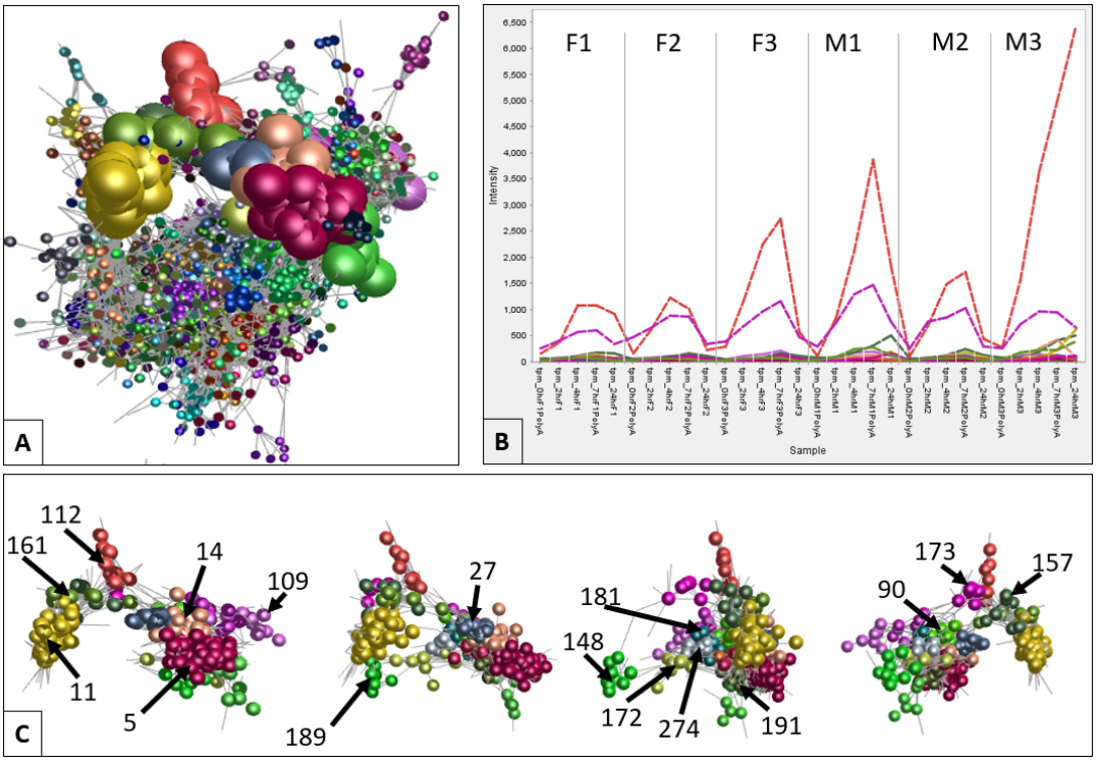
There was significant overlap between the gene lists of both pipelines, with the disparity due in part to the increasingly divergent responses between individuals. The largest, cluster 5, in which Male 3 showed the greatest level of expression (peaking at 4 hr), contains more genes associated with interferon signalling such as *STAT2, IRF7* and *IRF9* and *IFIT1, IFIT2, IFIT3, IFIT5* and *IFIT7*. This cluster contains a candidate novel IFN regulatory factor. *ENSOART00000001952* has both IFN regulatory factor-3 domain and SMAD/FHA domains and is lowly expressed. Based upon the nearest orthologues and comparison of syntenic regions using Ensembl (Aken *et al.*, 2016), it was found to share a 1:1 orthologue with no functional annotation with 9 other placental mammals, with shared synteny across the region and was listed as having 1:1 orthologues with various fish and aquatics, where it is annotated as *IRF10 (IRF10* does not exist in humans or mice and was first found in birds and is present in other tetrapods). This gene is also listed as a 1:1 orthologue with *IRF9* in various birds including the chicken (shared synteny). *IRF9* is already annotated in the sheep on a different chromosome (Chr 7), so this gene (which lies on Chr 13) has been assigned the annotation of *IRF9-like.*

Figure 5.10 Visualisation of the clusters demonstrating a regulated peak in response to LPS at 7hrs

A. Key clusters found to peak in expression at 7hrs in the wider network expression graph.

B. Mean expression of transcripts contained in each cluster across individuals: Female 1 (F1), Female 2 (F2), Female 3 (F3), Male 1 (M1), Male 2 (M2) and Male 3 (M3) and across time points 0hr, 2hr, 4hr, 7hr and 24 hr (left to right).

C. Clockwise rotation by 45 degrees of the clusters peaking at 7hrs.



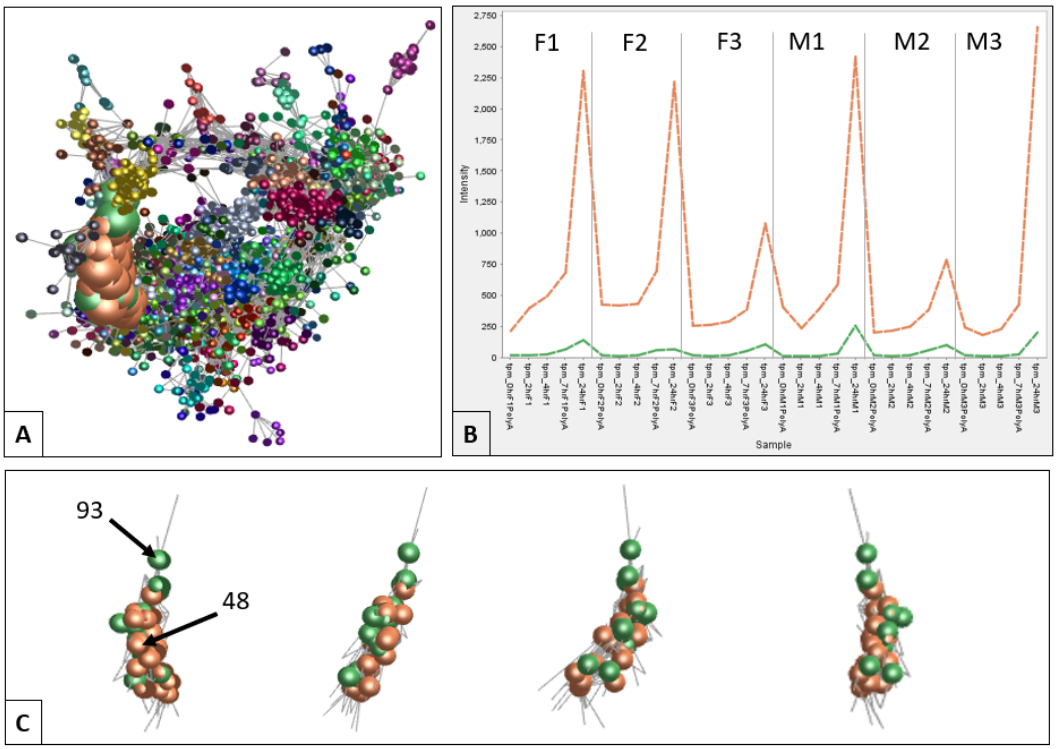
5.3.9 Inter-individual variation at the resolution of inflammation phase

Clusters which peak in expression at 24 hr after LPS stimulation in most of the individuals have been extracted from the network expression graphs (Figure 5.11) created using both Kallisto (Section 5.2.1) and StringTie (Section 5.2.2) with the individual values and can be viewed in Appendix 5.2 and 5.3 respectively.

There are relatively few resolution phase response clusters that increase in all individuals in the 24hr time point after LPS stimulation, reflecting the different positions of each animal at this time point in the PCA (Figure 5.1). The majority of transcripts centre around metabolism, encoding multiple solute carriers (See Section 5.5), S100 calcium binding proteins and for enzymes such as lactate dehydrogenase (LDHA), fatty acid desaturase 3 (FADS3) and matrix metallopeptidase 25 (MMP25), highlighting the metabolic progression

associated with the inflammatory response. The metabolic changes throughout the response are discussed in greater detail in Section 5.4.

Figure 5.11 Visualisation of the clusters demonstrating a regulated peak in response to LPS at 24hrs
A. Key clusters found to peak in expression at 24hrs in the wider network expression graph.
B. Mean expression of transcripts contained in each cluster across individuals: Female 1 (F1), Female 2 (F2), Female 3 (F3), Male 1 (M1), Male 2 (M2) and Male 3 (M3) and across time points 0hr, 2hr, 4hr, 7hr and 24hr (left to right).
C. Clockwise rotation by 45 degrees of clusters peaking at 24hr.



5.4 The metabolic shifts of sheep BMDMs in response to LPS

5.4.1 Metabolism in immune cells

Regulated intermediary metabolism is widely accepted as a key step in the differentiation and functional adaptation of cells of both innate and acquired immune systems (Murray *et al.*, 2015, O'Neill *et al.*, 2016, Gaber *et al.*, 2017). Circulating metabolic substrate concentrations are very different between ruminants and monogastric species. For that reason, there was a particular interest in this study on genes encoding membrane transporters and metabolic enzymes. Glucose, glutamine and fatty acids are all utilised as energy sources for mammalian macrophages. Glucose is utilised to generate ATP (by glycolysis and mitochondrial oxidative phosphorylation), glycerol-3-phosphate (for making

phospholipids and triacyglycerols), NADPH (to produce ROS) and ribose (for RNA synthesis) (Curi *et al.*, 2017).

Mouse and rat macrophages have been shown to metabolise glutamine at a rapid rate (Curi *et al.*, 2017). Inhibition of glutamine synthetase (GSS), which produces glutamine from glutamate, was shown to alter the polarisation state of macrophages (Palmieri *et al.*, 2017). The circulating glutamine concentration in ruminants is 3-5 fold lower than in monogastric species, due to a low glutamine synthetase capacity, and glutamine is not the predominant respiratory fuel for the intestine (Meijer *et al.*, 1993). When there is increased availability of other energy substrates, there is decreased glutamine utilisation in the intestinal wall (Reynolds & Huntington, 1988). In conditions of metabolic stress (such as high milk production in dairy cows), plasma and tissue glutamine pools are both similar to those of most essential amino acids such as methionine and lysine (Meijer *et al.*, 1993).

Fatty acids are also used by mouse macrophage mitochondria, for energy and in lipid synthesis. The phenotypic plasticity of macrophages is known to be influenced by metabolites including glucose, lipoproteins, fatty acids and many nutrients derived from the microbiome (O'Neill *et al.*, 2016, Stienstra *et al.*, 2017). The sheep, as a ruminant, has high circulating levels of fermentative by-products, primarily volatile fatty acids (propionate, acetate and butyrate), which are utilised within the liver for gluconeogenesis(Danfaer *et al.*, 1995). Aside from acting as fuels, free fatty acids may be recognised by specific G protein coupled receptors (FFAR1-4, GPR 84,35,91 and 109A) in cells of the immune system as well as tissues involved in metabolic function such as the pancreas and intestinal epithelium (Alvarez-Curto & Milligan, 2016).

Increased glucose metabolism in activated macrophages and disruption of the TCA cycle is proposed to be essential for the secretion of pro-inflammatory cytokines and production of ROS in mouse macrophages (Freemerman *et al.*, 2014, Jha *et al.*, 2015, Ryan & O'Neill, 2017). Aside from the generation of ATP and metabolites for macromolecule synthesis, regulated glycolytic enzymes such as GAPDH (Nagy & Rigby, 1995, Millet *et al.*, 2016), pyruvate kinase, lactate dehydrogenase, enolase and aldolase, are known to bind to mRNA and regulate protein synthesis (Castello *et al.*, 2012, Donnelly & Finlay, 2015). Hexokinase 1 (HK1) has also been shown to regulate NLRP3 inflammasome activation (Moon *et al.*, 2015).

Fatty acid synthesis in the cytosol, which also depends upon glucose metabolism, is also closely linked to macrophage effector functions (Feingold *et al.*, 2012, Tannahill *et al.*,

2013). For example, CSF1 up regulates genes required for fatty acid synthesis whilst driving the differentiation of human monocytes, with differentiation associated with a change in lipid synthesis from cholesterol (monocytes) to phophatidylcholine (macrophages) (Cader *et al.*, 2016). In this respect, human macrophages differ from mouse (Irvine *et al.*, 2009). Conversely, the beta oxidation of fatty acids has been associated with anti-inflammatory macrophages. Lipolysis may fuel mitochondrial oxidative phosphorylation leading to reduced ER stress, ROS damage and inflammation (Malandrino *et al.*, 2015).

In overview, circulating metabolites including products of the microbiome can influence the innate immune response (Yurkovetskiy *et al.*, 2015, Rooks & Garrett, 2016). The original hypothesis for this project (Section 1.7), suggested that ruminants may have distinct regulation of intermediary metabolism in their immune cells. This is likely due to the ruminant symbiotic fermentative microbiome and ruminant distinctive hepatic gluconeogenic metabolism which will alter homeostatic levels of circulating fatty acids and nutrients compared to monogastric species. Based upon that hypothesis, this Section is focussed specifically on the regulation of metabolism-associated genes in sheep macrophages.

5.4.2 Analysis of genes involved in intermediary metabolism

Because of the large number of reference transcripts expressed by sheep macrophages, the thresholds required for visualisation, and the variation in expression between individuals, many metabolic genes of interest were excluded from cluster analysis in previous Sections. To enable a comprehensive analysis, Kallisto expression estimates for the BMDM response (PolyA-selected) samples were averaged across all individuals. This average expression value was then extracted for all reference transcripts involved in intermediary metabolism. A list of genes with the primary annotation of Metabolic Process was downloaded from the MGI database (URL http://www.informatics.jax.org/vocab/gene_ontology/GO:0008152). A total of 7000 genes had secondary annotation indicating the likely process/pathway. That list was curated to contain only genes with a relevant secondary annotation related to intermediary metabolism. The curated list was supplemented with known genes of interest that have more recently been annotated, such as aconitate dehydratase (*ACOD1*) and genes involved in the uptake of nutrients, their transportation and metabolite conversion. All annotated solute carriers and cytochrome P450 (CYP) family members were included. Genes with more than one transcript variant in the reference appear twice. The ratio of the

maximum averaged expression and mean averaged expression of each transcript was used to identify genes which were most regulated during the LPS response. The final table can be viewed in Appendix 5.10.

This table showed that the majority of genes involved in intermediary metabolism were constitutively-expressed in macrophages and showed no evidence of immune cell enrichment compared to the wider sheep atlas (Clark *et al.*, 2017). The subset of genes constitutively highly-expressed by macrophages and macrophage-enriched compared to other tissues may enable rapid metabolic responses to stimulation. They include genes that encode the glucose transmembrane transporters, namely *SLC2A1* and *SLC2A6*, also known as *GLUT-6* (Fukuzumi *et al.*, 1996, Freemerman *et al.*, 2014), and a novel *leukocyte hexokinase 3 gene (HK3*). HK1 may have another function as a PRR (Wolf *et al.*, 2016) as well as having a critical role in the NLRP3 inflammasome (Moon *et al.*, 2015). It is not known whether HK3 has a similar function.

Aside from gene transcripts encoding the glucose transporter(s), gene transcripts encoding most of the glycolytic enzymes (e.g. *PKM, PFKL, PFKP, PGK1, PGM2/3, LDHA*) including those of the pentose pathway (*PGD, TADO1*) were found to be highly-expressed by sheep BMDM (>>100TPM). The transcript encoding the rate-limiting enzyme of the pentose pathway, which enables the production of NADPH for oxidative killing by the phagocyte oxidase, glucose-6-phosphate dehydrogenase (*G6PD* gene, annotated as *ENSOARG00000004237*), was also highly-expressed by sheep BMDM, alongside genes encoding the components of the phagocyte oxidase (*NCF1, CYBA, CYBB*) (Iles & Forman, 2002, Kim *et al.*, 2017). Constitutive high expression of genes involved in glucose metabolism in sheep BMDM may partially reflect the culture methods used in this project (Tavakoli *et al.*, 2017).

A second subset of the genes in Appendix 5.10 with a primary annotation of metabolic process were highly regulated by LPS. Genes encoding glucose transporters, *SLC2A1* (encoding GLUT1) and *SLC2A6* (encoding GLUT6) were further induced by LPS, the latter >10-fold within 2 hours. The same pattern is observed in mice and humans (Schroder *et al.*, 2012) and in all these species, *SLC2A6* expression is largely restricted to stimulated macrophages. The glycolytic activator 6-phosphofructose-2-kinase and fructose-2,6-bisphosphatase encoded by *PFKFB3* is proposed to upregulate glycolysis and link glucose metabolism to cell proliferation and survival in mouse macrophages (Jiang *et al.*, 2016).

PFKFB3 was highly-expressed and induced further by LPS. The gene transcript encoding Pyruvate kinase, muscle (*PKM* aka *PKM2)* and both transcripts encoding lactate dehydrogenase A *(LDHA*), as well as *SLC16A3 (*which encodes the monocaboxyate carrier MCT4) and *SLC16A6* were further elevated later in the response, after 24 hr. The TLR-inducible expression of SLC16A3 is shared with mice, and in that species mediates the export of lactate from glycolysis as part of a positive feedback mechanism (Tan *et al.*, 2015). SLC16A3 may also mediate uptake of the ketone body, acetoacetate, in exchange for lactate, but has a relatively low affinity for beta-hydroxybutyrate and short chain fatty acids (Dimmer *et al.*, 2000).

Although much of the flux of glucose through glycolysis gives rise to lactate, there was no evidence for the absolute loss of TCA cycle or mitochondrial gene expression in response to LPS. The set of transcripts that were partly down-regulated by LPS (see also Section 5.4.5 above), includes some encoding mitochondria-associated proteins, for example NDUF (NADH ubiquinone oxidoreductase) subunits FA10, FA2, FB4 and FB5, along with SDHC, but most genes associated with oxidative phosphorylation were unaffected.

Intermediates of the TCA cycle are known to provide precursors for other biosynthetic pathways. For example, TCA cycle intermediate metabolites function as checkpoints for multiple key LPS response genes, including *IL1B* (Jha *et al.*, 2015, Papathanassiu *et al.*, 2017) and *IL1B e*xpression has already been highlighted as particularly interesting in the sheep (see Section 5.3.7). The levels of citrate carrier (encoded by *SLC25A1*), which promotes the export of citrate from the mitochondria to the cytoplasm, can influence NO, ROS and PGE2 production in human macrophages (Infantino *et al.*, 2014). *SLC25A1* was induced in mouse macrophages in response to LPS, but in sheep BMDM, in common with pig, *SLC25A1* was repressed.

In the TCA cycle, citrate is initially converted to cis-aconitate by mitochondrial aconitase 2 (encoded by the gene transcript *ACO2*). In stimulated mouse macrophages, the TCA cycle is diverted through the induction of a novel enzyme, cis-aconitate decarboxylase 1, encoded by *immune responsive gene 1 (IRG1* also known as *ACOD1).* ACOD1 catalyses the conversion of cis-iconitate to cis-itaconate (Michelucci *et al.*, 2013, Diskin & Pålsson-McDermott, 2018). Cis-itaconate is a novel anti-inflammatory metabolite that exerts feedback inhibition of the inflammatory response (Jha *et al.*, 2015, Mills *et al.*, 2018). *ACO2* was robustly expressed across the sheep BMDM time course, but not specifically regulated

in response to LPS, similar to humans, mice and pigs. *ACOD1* was rapidly (by 4 hr) induced in response to LPS in all individual sheep but the induction and level of expression was low compared to mice; only reaching similar levels to aconitase 2 *(ACO2).* In mice, the induction of *ACOD1* leads indirectly to accumulation of downstream TCA cycle intermediates, succinate, fumarate and malate. Succinate may be an important metabolite in innate immune signalling which enhances IL1B production (Tannahill *et al.*, 2013). Interestingly, the G-protein-coupled receptor for succinate from the TCA cycle (encoded by *SUCNR1* also known as *GPR91*)(He *et al.*, 2004) was highly upregulated in LPS stimulated sheep macrophages, whereas it is undetectable in mouse BMDM. In mice, *Gpr91* is restricted to antigen-presenting dendritic cells (Rubic *et al.*, 2008). In humans, it has been suggested that *GPR91* is involved in renovascular hypertension, closely-related to atherosclerosis, diabetes and renal failure (He *et al.*, 2004). This fundamental difference between sheep and mice/humans may relate to production of succinate by rumen microorganisms (Kennedy *et al.*, 1991).

In other species, activated macrophages also use glutamine through anaplerosis to replenish TCA cycle intermediates (Costa Rosa *et al.*, 1991, Wallace & Keast, 1992, de Oliveira *et al.*, 2016, He *et al.*, 2016). Glutamine can also act as a nitrogen donor for nucleotide (purine and pyrimidine), and protein synthesis, and can be converted to glutamate, which in turn is widely utilised in multiple metabolic pathways (Young & Ajami, 2001). By contrast to mouse and human macrophages (where expression was low, and not regulated), in sheep BMDM *SLC1A5* (also called *ASCT2*), encoding the major glutamine transporter (Nicklin *et al.*, 2009, Liu *et al.*, 2015) was highly expressed and further induced by LPS. Knocking down this transporter in human PBMCs has been shown to interfere with IL1B expression (Tannahill *et al.*, 2013). Genes encoding two other transporters that were LPS-inducible in sheep BMDM, *SLC7A5* and *SLC7A11*, along with *SLC1A5*, have all been implicated in the so-called glutamine addiction of human cancer cells (Alexander *et al.*, 2017). Only *SLC7A11* appears to be similarly regulated by LPS in mice. This suggests that future studies could investigate the hypothesis that these transporters are likely to be the rate-limiting steps for glutamine metabolism within sheep BMDM. Both transcript variants for glutaminase (*GLS* gene), which catalyses the hydrolysis of glutamine to glutamate and ammonia as well as downstream genes in the glutamine pathway such as *GOT1/2, GLUD1/2, MDH* and *GLUL* were constitutively-expressed and not further regulated by LPS.

One notable feature of the sheep BMDM was the very highly expressed gene encoding L-asparaginase (*ASRGL1*), which was further up-regulated later in the LPS response. This gene in humans is expressed specifically in brain and testis and has been suggested as being involved in the production of L-aspartate (an excitatory neurotransmitter in some brain regions) (www.genecards.org),(Baslow, 2000). Asparaginase may also possess glutaminase activity (Chan *et al.*, 2014) and asparagine is likely taken up by *SLC1A5/ASCT2.* Weiske reported in 1879 that asparagine supports weight and a positive nitrogen balance in sheep (Ruckebusch & Thivend, 2012). Both asparagine and glutamine serve as nitrogen sources for the growth of rumen microbes, asparaginase mainly being associated with the bacteria, and glutaminase with the protozoa (Hoshino *et al.*, 1966). In humans, asparagine is a nontoxic carrier of residual ammonia to be eliminated from the body, and regulates the uptake of other amino acids, serine, arginine and histidine metabolism and thus protein and nucleotide synthesis (Krall *et al.*, 2016).

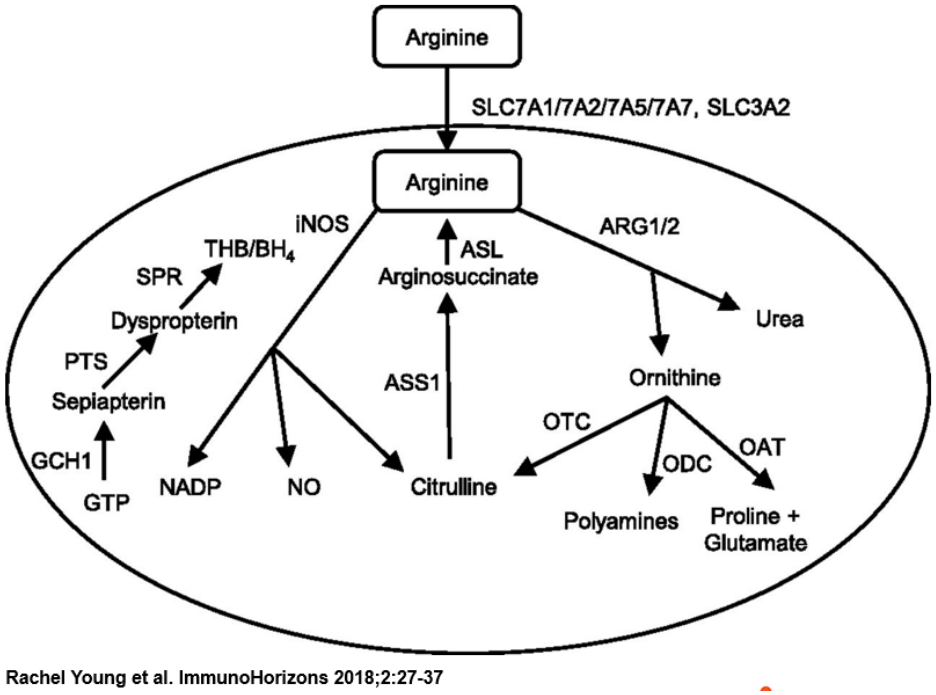
The production of nitric oxide also depends upon the uptake and intracellular availability of the NOS2 substrate, arginine. The data generated in this project contributed to a comparative study, involving multiple colleagues in the laboratory, of the regulation of the metabolism of arginine by LPS-stimulated BMDM in multiple species: sheep, goat, cattle, horse, water buffalo, human, pig and rat (Young *et al.*, 2018). The summarised data is shown in Table 5.5, with Figure 5.12. In addition to differences in *NOS2* induction, macrophages from sheep and other large animals expressed distinct sets of genes encoding amino acid transporters compared to mice, in particular lacking expression of transcripts encoding the LPS-inducible cationic amino acid transporters, *SLC7A1* and *SLC7A2* (Yeramian *et al.*, 2006, Wanasen *et al.*, 2007, Thompson *et al.*, 2008, Chaturvedi *et al.*, 2010). SLC7A2 is particularly important in the gastrointestinal tract of mice where is appears to regulate the attachment of intestinal pathogens and downstream signalling, recruitment of myeloid cells and the adaptive immune response (Singh *et al.*, 2016). Sheep express *SLC3A2/SLC7A7* constitutively , suggesting these may be the likely transporters for arginine. A protein which is also able to transport arginine, lysine and ornithine, encoded by *SLC7A3* (assigned to *ENSOART00000000566 / ENSOARG00000000528,*), was also induced in the sheep LPS response.

As shown in Fig 5.12, arginine may also be metabolised by alternative pathways. The balance between arginase, which promotes ornithine and urea production and therefore

pro-proliferation, repair and growth of parasitic and bacterial infections, and nitric oxide synthase (NOS) enzyme, which generates nitric oxide (NO) species required for intracellular killing of pathogens, has been shown in mice to dictate the course of any response (Bronte & Zanovello, 2005). There are at least two isoforms of mammalian arginase (ARG1 and ARG2). The induction of arginase 1, encoded by *ARG1* has been associated with alternative activation of mouse macrophages (Martinez *et al.*, 2013). In mice, *Mycobacterium tuberculosis* (Mtb) evades the innate immune response by manipulating macrophage production of antimycobacterial NO (Kaufmann *et al.*, 2005). Mtb may block the recruitment of NOS to the phagosomal membrane (Davis *et al.*, 2007), as well as increasing expression of ARG1 which creates competion for NOS for arginine, reducing NO production (Qualls *et al.*, 2010). In humans, ARG1 is produced in Mtb granuloma-associated macrophages and type II pneumocytes, not lymphocytes and ARG2 is not upregulated in human Mtb infected lungs (Pessanha *et al.*, 2012). The induction of ARG2 may be upregulated in macrophages infected by intracellular pathogens such as *Trypanosoma cruzi, Trypanosoma brucei* and *Helicobacter pylori* (Das *et al.*, 2010).

Table 5.5 summarises the data on expression of transcripts associated with arginine metabolism in multiple species. In common with other ruminants, sheep showed preferential inducible expression of *ARG2*, which encodes a mitochondrial arginase enzyme, which catalyses the hydrolysis of arginine to ornithine and urea, as part of the urea cycle, with no detectable increase in expression of *ARG1* during the macrophage response to LPS, and was contrary to the horse and pig where *ARG2* was downregulated in the response. These differences could explain why sheep are more susceptible to Toxoplasma gondii than cattle (Esteban-Redondo *et al.*, 1999). Sheep macrophages also expressed high levels of *ornithine amino transferase (OAT)* transcripts, encoding the downstream enzyme which leads to the production of glutamate. Sheep and goats (to a lesser extent) were the only species that increased expression of *ODC1,* encoding ornithine decarboxylase which gives rise to polyamines. In mice, ODC1 has also been characterised in the anti-inflammatory macrophage response to parasite and fungal infections (Seabra *et al.*, 2004, Heby *et al.*, 2007). The unique commitment of sheep macrophages to these pathways further highlights the importance of recycling of urea to rumen microorganisms in ruminants.

Figure 5.12 Mammalian arginine metabolism pathway in macrophages
Taken with permission from (Young *et al.*, 2018) to show the known genes involved in arginine metabolism



In the cluster analysis of the LPS time course (Section 5.3.7, peaking at 4hrs in response to LPS), *ARG2* and *ODC1* were co-expressed with *UPP1*, the gene for uridine phosphorylase which produces uracil and ribose- or deoxyribose-1-phosphate, used for energy, carbon and to rescue pyrimidine bases for nucleotide synthesis (www.genecards). *UPP1* is already known to have associations to inflammatory cytokines and the immune response (Watanabe & Uchida, 1995, De Santa *et al.*, 2009).

Table 5.5 Differential expression patterns of enzymes associated with arginine metabolism and the production of NO during the macrophage response to LPS.
The table is simplified from expression data gathered by Young et al, with all domestic animal data generated by RNA-Seq and human data was generated by CAGE seq, described within the paper (Young *et al.*, 2018). Where genes had a TPM <1 for both the unstimulated (0hr) and LPS stimulated (7hr) they are displayed as 0. Where the differential expression between samples was <5TPM, it has been deemed as 'no change' and displayed as nc. Nitric oxide (NO) production was assessed using a Griess assay, for which I contributed the sheep data. In response to LPS, Sheep and Horse macrophages do not produce NO, goat and buffalo macrophages produce small amounts of NO and both cattle and chicken (not shown) macrophages produce similarly large amounts of NO, as indicated by the approximate maximum nitrite concentration (µM) taken from the paper (Young *et al.*, 2018). Pig, rat and human griess assay results were not available (n/a), however it is recognised that pig and human macrophages do not produce NO, and rat macrophages, like mice produce large amounts of NO in response to LPS (Kapetanovic *et al.*, 2012).

Gene name	Sheep	Goat	Cattle	Buffalo	Horse	Pig	Rat	Human
ARG1	0	nc	nc	0	0	↑↑	↑↑↑	0
ARG2	↑	↑↑	↑↑	↑↑	↓↓	↓	nc	nc
ASL	↓	↓	↓	↓	↓	↓	↓	↓
ASS1	↓	nc	↓	0	↓	0	↑↑↑	0
GCH1	↑	nc	↑	nc	↑	nc	↑↑	↑↑↑
NOS2	↑	↑	↑↑↑	↑↑	0	nc	↑↑↑↑	0
OAT	↓	nc	↓	↓	↓	↓	↓	↓
ODC1	↑↑	↑	↓	↓	↓	↓	↓	nc
PTS	nc	nc	↓	↓	↓	nc	nc	nc
SLC3A2	↑	nc	↓	↓	↓	↑	↑	↑
SLC7A1	nc	↑	nc	↑	nc	nc	↓	0
SLC7A2	0	↑	0	nc	0	nc	↑↑↑	0
SLC7A5	↑	↓	nc	nc	nc	nc	nc	↑↑
SLC7A7	↓	↓	↓	↓	↓	↑	nc	↓↓
SPR	↓	↑	↓	↓	↓	↓	nc	↓
NO production	0.1	6	17	6	1	n/a	n/a	n/a

In humans and pigs, which also do not make NO, an alternative effector pathway, involving the metabolism of tryptophan by the inducible enzyme indoleamine dioxygenase (IDO1) is strongly induced by LPS (Kapetanovic *et al.*, 2012). IDO1 has been implicated in host defence against mycobacteria, with increased IDO levels in affected tissues corresponding to progression to clinical mycobacterial disease (Plain *et al.*, 2011); and chlamydia, where

intracellular pools of tryptophan are degraded by IDO in response to IFN-gamma and arrest growth of the organism (Entrican *et al.*, 2009). Surprisingly, there was no detectable induction of *IDO1* mRNA in the LPS-stimulated sheep BMDM, whereas the transcript was detected in other immune tissues in the atlas, and downstream genes in the tryptophan pathway, *KYNU* and *KMO* were robustly expressed and further induced by LPS. Since IDO1 is interferon-responsive in other species (Desvignes & Ernst, 2009, Kane *et al.*, 2016), it may be that induction in sheep macrophage requires priming by IFN-gamma.

Fatty acid oxidation is highly efficient at generating ATP (O'Neill *et al.*, 2016). Sheep macrophages do appear to be well-adapted to use free fatty acids as fuels, perhaps reflecting their availability in the ruminant circulation. Multiple genes encoding lipases (*LPL, LIPA*) and cytoplasmic fatty acid binding proteins (*FABP4, FABP5*) were expressed at exceptionally high levels (>1,000 TPM). Most enzymes required for fatty acid oxidation were constitutively expressed, and not regulated by LPS. The gene encoding the key transcriptional regulator of peroxisomal beta-oxidation pathway of fatty acids, *peroxisome proliferator activated receptor delta (PPARD)*, was also constitutively-expressed, but further induced by LPS. PPARD is a dietary lipid sensor which has a preference for poly-unsaturated fatty acids, but depending upon the context, activation induces both anti-inflammatory and specific stimulatory molecules, including suppressing release of kynurenine (Adhikary *et al.*, 2015). The gene encoding the receptor for medium chain free fatty acids (C9-C14), *GPR84*, was also highly induced in response to LPS in sheep, as it is in mice, pigs and humans. As noted with reference to arginine metabolism, ruminant and other large animal macrophages expressed distinct sets of amino acid transporters compared to mice. The increased expression of nucleoside transporter gene *SLC28A3,* in response to LPS, also appears to be specific to sheep, with no detectable expression in mouse macrophages and a slight decrease in expression in response to LPS in human macrophages.

5.5 Discussion

Genes associated with immunity are under strong evolutionary selection (as discussed in Section 1.1). The hypothesis underlying this project was that sheep macrophages may have evolved novel mechanisms and patterns of gene expression in response to an immune challenge such as LPS. As seen in other animals, LPS initiates a cascade of gene expression following TLR4 ligation. As expected, the transcriptome of BMDM in the sheep is

remarkably diverse with 85% of the reference transcriptome detected above a conservative threshold over the response to LPS (see Table 5.1). The transcriptome of stimulated macrophages is similarly rich in the mouse (Wells *et al.*, 2003) and accordingly, macrophage RNA-Seq data makes a major contribution to genome annotation. Based upon their regulated expression, hundreds of genes identifiable only by Ensembl ID were manually assigned a functional annotation, including genes for transcription factors such as EGR1, EGR4, KLF2; cell surface receptors such as CD36, PTGIR; signalling molecules (PYCARD), transporters such as SLC25A30 and numerous chemokines, cytokines and enzymes.

Four different network approaches were used to explore the BMDM response to LPS over a 24 hr time course. Firstly, an analysis of the macrophage specific clusters within a network of all samples described in The Sheep Atlas (Clark *et al.*, 2017) using averaged values, provided a comparison of the stimulated sheep macrophage signature in relation to the wide range of other cell types and tissues available within the atlas (Section 5.4.2). A small number of genes were identified that were found to vary in expression pattern compared to other species (Table 5.3).

Secondly, also using averaged values, all immune cell samples were clustered (Section 4.3.2, Appendix 4.1) and the BMDM response specific clusters were identified, in order to extract the set of transcripts that were macrophage and LPS response-specific relative to other immune cells. Consistent with the core hypothesis, a number of genes were uniquely expressed and regulated in sheep macrophages (Table 5.4).

Thirdly, the majority of the analysis focussed on expression values for individual sheep generated by the Kallisto pipeline. Evolutionary selection acts upon individual variation within any given population, and one would also anticipate that individual sheep differ from each other, especially between breeds subjected to divergent selection. In humans, the large majority of genes expressed in human macrophages in response to LPS exhibited heritable variation in the level of expression (Fairfax *et al.*, 2014), and such variation has been associated with susceptibility to inflammatory bowel disease (Baillie *et al.*, 2017). In pigs, variation between individuals was also observed, whereas there was no consistent impact of breed (Kapetanovic *et al.*, 2013). This study compared the response of 6 cross-bred sheep. There was substantial variation between individuals that reinforced the identification of sets of co-regulated genes. Female 1 appeared to give a rapid early response and regain near-basal levels of expression within 24 hrs, whereas male 3 failed to

switch off an abundance of interferon responsive genes including those encoding the inducible transcription factors, *IRF1, IRF5* or *IRF8.*

The Kallisto results were validated by examining a network analysis created using expression values generated by the StringTie pipeline for each individual. Numerous novel splice variants and potential novel long non-coding RNA transcripts were revealed.

Finally, the expression and regulation of metabolism-associated transcripts in sheep BMDMs was explored in detail. This analysis highlighted those that are highly regulated and/or highly expressed during the BMDM response in sheep. The high expression of *SLC1A5,* appeared to be novel and specific to sheep. The high expression profile of *ARG2* is shared with other ruminants, and some gene profiles are only shared with closely related species, namely the goat, such as *ODC1.* The regulated expression of *ARG2* in sheep (shared with other ruminants and horses) results from evolutionary divergence of promotor sequences compared to other large and small animal species, notably the insertion of a retrotransposon (BOV-A2) around -3 kb in sheep and goats (Young *et al.*, 2018). Altering the promotor architecture can result in the gain or loss of enhancers (Kapetanovic *et al.*, 2012, Schroder *et al.*, 2012). Similarly, a BOV-A2 insertion distinguishes the NOS2 promoters of cattle and water buffalo from sheep and goat, and may explain the differential LPS inducibility, as it contains binding sites for many macrophage specific (PU.1, CEBPβ) and inducible (STAT1, IRF1, NFKB) transcription factors (Young *et al.*, 2018). As discussed in Section 5.4.7, sheep macrophages did not produce detectable nitric oxide in response to LPS, presumably a consequence of high *ARG2* as well as low *NOS2* induction. In stimulated mouse macrophages the production of nitric oxide suppresses respiration through the inhibitory effect on haem-containing components of the electron transport chain (Van den Bossche *et al.*, 2016). Since sheep macrophages do not produce nitric oxide, and also have relatively low expression of *ACOD2,* they are unlikely to exhibit the decreased respiration and "broken" TCA cycle reported in activated mouse macrophages (Mills & O'Neill, 2016).

Ruminant and/or sheep/goat-specific regulation could reflect the evolution of the rumen in providing optimal conditions for microbial fermentation and the wide variety of microbes, many of which cannot be cultured in a lab (Krause & Russell, 1996). Future laboratory work is needed to determine whether the retention of a fully functional TCA cycle allows sheep

macrophages to utilise fatty acids that are a major circulating product of ruminant digestion as fuels.

The seven-transmembrane/ G-protein coupled receptors are a group of receptors, involving G-protein coupling and activation of second messenger generating enzymes, and are known to have remarkably diverse biological functions, with members evolving rapidly (Pierce *et al.*, 2002, Kwakkenbos *et al.*, 2004, Hume, 2006), so it was anticipated that the sheep may have different functional members and possibly novel members for themselves. Free fatty acids receptor (FFARs) act as physiological sensors of food-derived FFAs and digestion products in GIT, thus they are important for nutrition and act as signalling molecules in numerous physiological processes (Hara *et al.*, 2013).

In ruminants, a wide variety of circulating FFA is indicative of health whereas in non-ruminants the same metabolites would be pro-atherogenic (Netea *et al.*, 2016, Groh *et al.*, 2017), being recognised as DAMPs by the innate immune system. In mice at least, FFAR-2 has been implicated in communication between the intestinal microbiome and the microglia of the brain (Erny *et al.*, 2015). Induction of the gene encoding a FFA receptor, *GPR84*, in sheep macrophages was shared with other species, but in sheep it may have access to a much higher concentration of ligand in the circulation. The response of sheep macrophages to added FFA is clearly worthy of future study.

One novel GPCR expressed in sheep macrophages, and highly-regulated by LPS, is adenylate cyclase activating polypeptide 1 (Pituitary) Receptor (*ADCYAP1R1* gene). This gene was not expressed or regulated in human, mouse and pig macrophages. *ADCYAP1R1* shares significant homology with members of the glucagon/secretin receptor family and the receptor is known to regulate the release of adrenocorticotropin, luteinizing hormone, growth hormone, prolactin, epinephrine, and catecholamine (Ogi *et al.*, 1993, Yon *et al.*, 1998, Vaudry *et al.*, 2000). The gene is predominantly expressed in the central nervous system in humans, mice and pigs. In the sheep atlas, the LPS-stimulated level of expression is comparable to that of the sheep brain and adrenal. In other species, the ligand, ADCYAP1, appears to be brain-restricted, but the ligand gene is not currently annotated in Ensembl, so it is not possible to assess whether it might have distinct functions in sheep.

Another macrophage-specific and LPS-regulated transcript of wider interest to macrophage biologists is *ADGRE1*, which in mouse encodes the macrophage-specific F4/80 antigen (Hume *et al.*, 1984). The sheep RNA-Seq data generated herein was used in a comparative

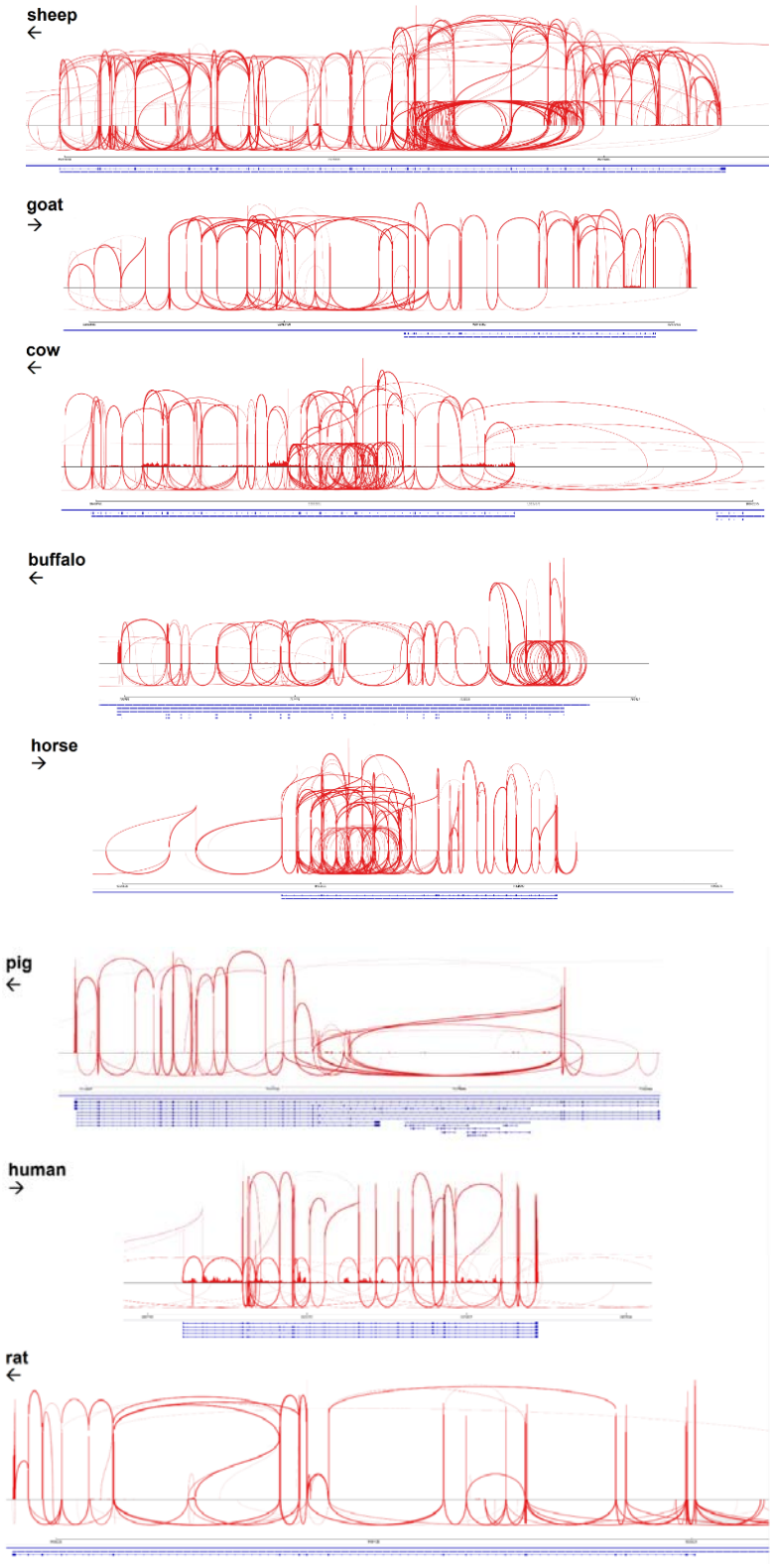
analysis of gene expression data from multiple species (Waddell *et al.*, 2018). That analysis revealed significant divergence in both basal expression and induction by LPS amongst species (Appendix 5.11, taken from manuscript (Waddell *et al.*, 2018)). The mouse *Adgre1* gene encodes a GPCR with an extracellular region containing 7 EGF-like calcium-binding domains, which are subject to extensive alternative splicing. The sheep macrophage RNA-Seq data revealed that the sheep *ADGRE1* locus contains a complete internal duplication of the extracellular domain, encoding 14 EGF-like domains, with each subject to alternative splicing/skipping. Figure 5.13 shows the Sashimi plots for the sheep RNA-Seq data, derived from the manuscript, compared with a number of other species that appear to share the duplicated extracellular domain (Waddell *et al.*, 2018). The extensive exon skipping is most evident in the sheep profile because of the much greater depth of sequencing. The manuscript discusses the possibility that *ADGRE1* encodes a pattern recognition receptor.

The analysis of macrophage-specific and LPS-inducible regulation in this chapter has not considered the long non-coding RNAs (lncRNAs). These are a significant component of the LPS-inducible transcriptome in both mouse and human macrophages; some of them associated with transcripts derived from regulated enhancers (Melamed *et al.*, 2016, Baillie *et al.*, 2017). Because of their low expression, lncRNA are difficult to quantitate and to assemble reliably from short-read RNA-Seq data and most fell below expression threshholds used here. The macrophage data, alongside the wide sheep atlas and a parallel study of goat and published cattle and human data was used in cross-species inference of lncRNA in ruminants (Bush *et al.*, 2018). The consensus lncRNA set will permit future analysis of this component of the transcriptome in sheep macrophages.

It is beyond the scope of this thesis to discuss the function of every ruminant or sheep-specific transcript. Examples were summarised in Tables 5.3 and 5.4, and others, such the gene duplication of *SERPINB2*, were discussed briefly in context as they arose in the analysis. In overview, the core hypothesis, that the core sheep macrophage transcriptome, and the response to LPS, would exhibit species-specific features, was clearly strongly supported by the analysis of the transcriptome.

Fig 5.13 Sashimi plots for *ADGRE1* across multiple species (taken from submitted manuscript)

The large amount of splicing results in an abundant number of different isoforms, as every exon encoding an EGF-like domain can be skipped by alternative splicing in all species.



Chapter 6 Summary of achievements and future directions

6.1 General discussion

This thesis aimed to identify and annotate genes that are associated with the innate immune functions of macrophages in sheep. The analysis was based upon the development of a cell culture system to generate and characterise large numbers of macrophages from sheep bone marrow (BMDM) (Sections 2.3.1 and 2.4). The BMDM responded to the TLR4 agonist, LPS, as evidenced by release of the proinflammatory cytokine, TNF (Section 5.4.2 and 5.3.6). Using this system, bone marrow from six healthy male (n=3) and female (n=3) adult sheep (BF X T) was used to generate BMDM and to produce RNA-Seq (both polyA-selected (mRNA) and ribodepleted (Total RNA)) data describing the response to LPS across a time course (Chapter 5). The RNA-Seq data were processed using two independent pipelines (StringTie and Kallisto), to determine genome wide transcript expression estimates for each sample. The data generated in this project made a significant contribution to the sheep transcriptional atlas (Section 5.3.2 (Clark *et al.*, 2017)). With nearly 90% of all nuclear encoded protein coding genes being detected in at least one BMDM sample at a given time point, the sheep BMDM response to LPS data provided the expected (Ravasi *et al.*, 2007) diverse and rich source of transcripts for study, and the data created (made available to Ensembl), will greatly improve future versions and functional annotation of the reference transcriptome.

The availability of the large sheep atlas dataset enabled a comparative analysis to identify transcripts that were enriched in macrophages and/or specifically associated with activation (5.3.2 and 5.3.3). Clustering transcript expression estimates using Miru, as other projects have done (Freeman *et al.*, 2012, Mabbott *et al.*, 2013, Raza *et al.*, 2014, Clark *et al.*, 2017), segregated the massive dataset into clusters containing transcripts that share similar patterns of expression. Based upon the principle of guilt-by-association (Oliver, 2000) the products of these transcripts are likely to share functions. Co-expression in macrophages with genes of known function provided the basis for prioritisation of genes for detailed annotation. Hundreds of genes possessing only an Ensembl ID were manually assigned a functional annotation based upon nearest orthologues and shared synteny with mouse or human (Section 2.11.1 for methods, Appendix 6.1 contains the full list of Ensembl IDs that have been annotated).

Publicly available datasets were used to compare the sheep macrophage gene expression profiles with those generated for monogastric animals; human (Baillie *et al.*, 2017), mouse (Schroder *et al.*, 2012), pig (Kapetanovic *et al.*, 2013) and cow (personal communications with Dr Rachel Young). Many of these differences between sheep and other species may be attributed in part to evolution of their relationship with the fermentative microbiome and increased levels of circulating fatty acids. The current view of the association of immune and metabolic genes with innate immunity is dominated by studies of mice and human cells. Evolution has provided the ruminant animal macrophage with a unique ability to function in a microenvironment that by human standards would be associated with the production of foam cells and atherosclerosis. Indeed, many of the hallmarks of foam cells were found in the resting sheep macrophage transcriptome signature. The predicted differences in intermediary metabolism between sheep and other species, based upon RNA-Seq data, now need to be confirmed using proteomic and metabolomic analysis of sheep macrophages (starting with TCA cycle intermediate investigations) using methods that have been applied previously only to the mouse (discussed in Section 5.4.2).

6.2 Genome annotation and the new genome assembly

Two pipelines were used to quantitate gene expression based upon mapping to the current reference genome OarV3 (Jiang *et al.*, 2014). A preliminary examination of LTR repeat regions, revealed variation between individual sheep in differential expression profiles between 0hr and 7hr, with BTLTR1 (a known ruminant specific LTR-containing repeat region (Elsik *et al.*, 2009). BTLTR1 was found to be the most regulated repeat region, decreasing in response to LPS (Section 3.6). OarV3.1 still contains many scaffolds, which will have affected the accuracy in mapping reads to repeat regions, but with the imminent release of the new genome assembly, which contains long read technology, many of these assembly issues will be resolved. Immediate future plans involve repeating the mapping of all RNA-Seq data from this project to the improved reference genome, Rambouillet v0.1 (https://www.ncbi.nlm.nih.gov/genome/83?genome_assembly_id=351950), which is a new highly contiguous PacBio assembly, incorporating many of the scaffolds that exist in OarV3.1. The accuracy of determining expression over these important areas of the genome will be greatly improved and future plans involve repeating this examination for all the classes of repeat regions, especially given the global interest surrounding Jaagsiekte sheep retrovirus (JSRV), which causes a pulmonary adenocarcinoma in sheep and goats (Rai *et al.*, 2001, Hofacre & Fan, 2010, Martineau *et al.*, 2011).

There is still RNA available from many of the samples that were submitted to the sheep atlas, including the BMDM time courses. Given the decreasing cost of sequencing, it may be possible to submit the samples for the PacBio Iso-Seq protocol. A recent report described a rabbit transcriptional atlas based upon long read sequencing (Chen *et al.*, 2017). Full length RNA sequences will improve assessment of the full diversity of alternative splicing of mature transcripts, especially for complex, multi exon genes of interest expressed in macrophages such as *ADGRE1, ADCYAP1R1* and *ITGAM* (Sections 5.5, Figure 5.13). Continued exploration of the novel and un-annotated transcripts identified in this project as being important in the sheep macrophage response will reveal many more novel transcripts to complement those identified herein. Long read RNA-Sequencing will also improve the identification of long non-coding RNAs. The sheep atlas, including the large macrophage dataset generated in this project, contributed to identification of many novel candidate lncRNA (Bush *et al.*, 2018), but their assembly from short-read data is challenging.

6.3 The diversity of macrophage responses

This study has examined the response of sheep BMDMs to a selective TLR4 agonist, LPS from *Salmonella enterica* serotype Minnesota Re 595. There remain many other forms of LPS, other TLR agonists and intracellular stimuli including specific pathogen challenge, to fully appreciate the response of sheep macrophages and individual variation in that response, as well as assessment of different breeds of sheep in their response. The response to mycobacteria would be especially interesting. Recent reports have contrasted the transcriptomic response in bovine alveolar macrophages and both the transcriptomic and proteomic response to *M. tuberculosis* and *M. bovis* (Malone *et al.*, 2018) and of monocyte-derived macrophages to *M. avium subsp paratuberculosis* (Marino *et al.*, 2017). Sheep are comparatively resistant to both of these infectious agents. Responses to other stimuli also differ between species. For example, in the response to alternative activation by IL-4, very few inducible genes were shared between mice and humans (Martinez *et al.*, 2013). Mice and humans vary in their response to glucocorticoids owing to enhancer divergence and the rapid evolution in the response between the species (Jubb *et al.*, 2016).

Aside from mycobacteria, many other pathogens are of particular concern to sheep health, welfare and productivity. Some are zoonotic and pose significant threat to humans or other production animals. Ongoing comparative analysis will help to identify host

determinants that underlie the differential pathology. To this end, the BMDM culture system employed in this project has been applied in the Hume laboratory to the cow, goat, rat, water buffalo (personal communication, Professor David Hume, University of Edinburgh), pig (Kapetanovic *et al.*, 2012), human (Baillie *et al.*, 2017), and mouse (Schroder *et al.*, 2012). Some of the differences between these species were analysed in Section 5.4.2 and were published in analysis of genes involved in arginine metabolism (Young *et al.*, 2018). A second study in the same set of species compares expression and evolution of *ADGRE1* (Section 5.5) (Waddell et al. submitted). For each of the genes identified as demonstrating a distinctive expression pattern to other animals, the obvious question is whether there is a specific function in host-specific pathogen recognition. For example, based upon comparative analysis (Section 5.4.2) nitric oxide production is unlikely to be a major component of anti-microbial defences in sheep and goats, or in horses, pigs and humans, where it is clearly important in rodents and possibly in large ruminants.

6.4 Individual variation in the response to LPS

An expression QTL analysis in humans revealed that >80% of LPS-inducible genes in monocytes exhibited heritable variation in the level of expression (Fairfax *et al.*, 2014). The six individual sheep were found to vary markedly in the response of their BMDMs to LPS: Male 3 appeared hyper-responsive whereas Female 1 responded more rapidly but transiently. The differences appeared to focus around interferon target genes (Section 5.3.7). A previous larger comparative study in pigs showed similar variation amongst individuals but did not find any evidence of breed-specific differences (Kapetanovic *et al.*, 2013). The cell culture system developed for this project, which utilises frozen bone marrow cells, can potentially be applied to much larger numbers of animals. It will be of great interest to determine whether the degree of variability of response observed in this study is fortuitous, or is representative of the population as a whole. Increasing the numbers of both male and female would also permit analysis of whether the sex of sheep influences the response, and which genes (if any) are sex specific in the response.

The animals used in this study were F1 crosses from two disparate breeds: the Texel and the Scottish Blackface. The high level of heterozygosity may actually have masked individual variation in the response but was intended to maximise the numbers of expressed SNPs. Future studies are required to utilise a much greater number of animals to determine if the degree of individual variation in response to LPS that has been noted in

this project, is present at the population level. One major future direction is to utilise the genomic sequence data available for each individual and examine the extent of allele specific expression across this dataset, as has been done in human (Knight, 2004, Ardlie K, 2015), mice (Crowley *et al.*, 2015) and cows (Chamberlain *et al.*, 2015). In outbred cattle, 89% of the genes tested had evidence of allelic imbalance in expression in at least one tissue, and as many as 28% of transcripts showed absolute monoallelic expression (Chamberlain *et al.*, 2015). Having identified the genes which are expressed differentially between alleles, it should be possible to infer the parent of origin, as the current reference genome is based upon a Texel, the sire breed of all the animals used in this project. A future direction will be to use the same procedure to examine both pure-bred animals, F1 crosses with the male and female parents reversed to confirm any evidence of parental imprinting, and F2 generation to begin to assess heritability. The heritability of gene expression will undoubtedly be complex, involving not only Mendelian genetic factors but parental metabolic states (Palu *et al.*, 2017).

Genes expressed in macrophages may also differ at the level of protein sequence. Current projects in Roslin and elsewhere are producing hundreds of genomic DNA sequences from sheep, goats, cattle, water buffalo and African buffalo, including those adapted to tropical environments with high disease burdens (Borriello *et al.*, 2006, Wang *et al.*, 2016, Banos *et al.*, 2017, Bhuiyan *et al.*, 2017, Wilkinson *et al.*, 2017). The prediction *a priori* is that functional variation in macrophage-expressed genes between individuals, and between breeds adapted to different environments, and between species, are more likely to be causally associated with differences in disease susceptibility.

6.5 Conclusion

The work described in this thesis has made several contributions to the understanding of macrophage biology. It has resulted in a dataset now in the public domain that can continue to be mined for insights, as indicated throughout the presentation of the results, where examples of information that can be derived from the data are shown. It has also provided a comprehensive analysis of the transcriptomic response to a cellular state change, highlighting the role of metabolic adjustments as macrophages respond to pathogen challenge. Adding the sheep data to that for other ruminant and monogastric animals has allowed a comparison which highlights the adaptations of the ruminant to maintain the critical balance with the microbiota of the gut. It opens the way to

understanding of the genetic basis of individual variation in immune response, both in sheep and more generally in other animals including humans. This work therefore has the potential to impact on human and animal health as well as production and food sustainability, key goals for research in the 21st century.

Appendices

Appendices are in files contained in the CD on the inside back cover of this thesis.

Appendix 2.1 (Edinburgh Genomics sample submission)

Appendix 3.1 (Kallisto v StringTie alignment rates)

Appendix 3.2 (SAMtools flagstat analysis)

Appendix 4.0 (StringTie and Kallisto expression estimates)

Appendix 4.1 (Kallisto immune cell clusters)

Appendix 5.1 (Sheep Atlas LPS response clusters)

Appendix 5.2 (Peaking Kallisto analysis)

Appendix 5.3 (Peaking StringTie analysis)

Appendix 5.4 (Kallisto PolyA LPS timecourse)

Appendix 5.5 (Final Kallisto PolyA post filtering)

Appendix 5.6 (Corrected StringTie PolyA samples)

Appendix 5.7(Kallisto Miru files)

Appendix 5.8 (Final StringTie PolyA post filtering)

Appendix 5.9 (StringTie Miru files)

Appendix 5.10 (Metabolism genes of interest)

Appendix 5.11 (ADGRE1 and ADGRE4 species comparison, taken from manuscript)

References

(2015) A complex cell. *Nature immunology* **17**: 1.
Adams DO (1989) Molecular interactions in macrophage activation. *Immunology today* **10**: 33-35.
Adams DO & Hamilton TA (1984) The cell biology of macrophage activation. *Annual review of immunology* **2**: 283-318.
Adelson DL (2008) Insights and applications from sequencing the bovine genome. *Reproduction, fertility, and development* **20**: 54-60.
Adelson DL, Raison JM & Edgar RC (2009) Characterization and distribution of retrotransposons and simple sequence repeats in the bovine genome. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 12855-12860.
Adhikary T, Wortmann A, Schumann T*, et al.* (2015) The transcriptional PPARβ/δ network in human macrophages defines a unique agonist-induced activation state. *Nucleic acids research* **43**: 5033-5051.
Aken BL, Ayling S, Barrell D*, et al.* (2016) The Ensembl gene annotation system. *Database (Oxford)* **2016**.
Akira S & Takeda K (2004) Toll-like receptor signalling. *Nature reviews Immunology* **4**: 499-511.
Akira S, Takeda K & Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology* **2**: 675-680.
Akira S, Uematsu S & Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* **124**: 783-801.
Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P (2002) Lymphocytes and the cellular basis of adaptive immunity.
Alexander SPH, Kelly E, Marrion NV*, et al.* (2017) THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: Transporters. *British Journal of Pharmacology* **174**: S360-S446.
Allison AC (1954) Protection afforded by sickle-cell trait against subtertian malareal infection. *British medical journal* **1**: 290-294.
Alvarez-Curto E & Milligan G (2016) Metabolism meets immunity: The role of free fatty acid receptors in the immune system. *Biochemical pharmacology* **114**: 3-13.
Amit I, Winter DR & Jung S (2016) The role of the local environment and epigenetics in shaping macrophage identity and their effect on tissue homeostasis. *Nature immunology* **17**: 18-25.
Andersson L, Archibald AL, Bottema CD*, et al.* (2015) Coordinated international action to accelerate genome-to-phenome with FAANG, the Functional Annotation of Animal Genomes project. *Genome biology* **16**: 57.
Andrews S (2010) FastQC A Quality Control tool for High Throughput Sequence Data. p.^pp.
Anwar MA, Basith S & Choi S (2013) Negative regulatory approaches to the attenuation of Toll-like receptor signaling. *Experimental & molecular medicine* **45**: e11.
Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL & Schwartz DA (2000) TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature genetics* **25**: 187-191.
Ardlie K DLea, G. TEx Consortium (2015) The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue gene regulation in humans. *Science (New York, NY)* **348**: 648-660.

Ashburner M, Ball CA, Blake JA*, et al.* (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature genetics* **25**: 25-29.
Austyn JM & Gordon S (1981) F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *European journal of immunology* **11**: 805-815.
Bahcall O (2014) Sheep genome. *Nature genetics* **46**: 665.
Baillie JK, Arner E, Daub C*, et al.* (2017) Analysis of the human monocyte-derived macrophage transcriptome and response to lipopolysaccharide provides new insights into genetic aetiology of inflammatory bowel disease. *PLoS genetics* **13**: e1006641.
Bain CC, Bravo-Blas A, Scott CL, Perdiguero EG, Geissmann F, Henri S, Malissen B, Osborne LC, Artis D & Mowat AM (2014) Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nature immunology* **15**: 929-937.
Baldi P & Hatfield GW (2002) *DNA Microarrays and Gene Expression: From Experiments to Data Analysis and Modeling*. Cambridge University Press, Cambridge.
Banos G, Bramis G, Bush SJ, Clark EL, McCulloch MEB, Smith J, Schulze G, Arsenos G, Hume DA & Psifidi A (2017) The genomic architecture of mastitis resistance in dairy sheep. *BMC genomics* **18**: 624.
Baranano DE, Wolosker H, Bae BI, Barrow RK, Snyder SH & Ferris CD (2000) A mammalian iron ATPase induced by iron. *The Journal of biological chemistry* **275**: 15166-15173.
Bartocci A, Mastrogiannis DS, Migliorati G, Stockert RJ, Wolkoff AW & Stanley ER (1987) Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 6179-6183.
Barua M, Stellacci E, Stella L*, et al.* (2014) Mutations in PAX2 associate with adult-onset FSGS. *Journal of the American Society of Nephrology : JASN* **25**: 1942-1953.
Baseler WA, Davies LC, Quigley L, Ridnour LA, Weiss JM, Hussain SP, Wink DA & McVicar DW (2016) Autocrine IL-10 functions as a rheostat for M1 macrophage glycolytic commitment by tuning nitric oxide production. *Redox biology* **10**: 12-23.
Baslow MH (2000) Functions of N-acetyl-L-aspartate and N-acetyl-L-aspartylglutamate in the vertebrate brain: role in glial cell-specific signaling. *Journal of neurochemistry* **75**: 453-459.
Basta G, Sironi AM, Lazzerini G, Del Turco S, Buzzigoli E, Casolaro A, Natali A, Ferrannini E & Gastaldelli A (2006) Circulating soluble receptor for advanced glycation end products is inversely associated with glycemic control and S100A12 protein. *The Journal of clinical endocrinology and metabolism* **91**: 4628-4634.
Batista FD & Harwood NE (2009) The who, how and where of antigen presentation to B cells. *Nature Reviews Immunology* **9**: 15.
Baumann CL, Aspalter IM, Sharif O*, et al.* (2010) CD14 is a coreceptor of Toll-like receptors 7 and 9. *The Journal of experimental medicine* **207**: 2689-2701.
Belinda LW, Wei WX, Hanh BT, Lei LX, Bow H & Ling DJ (2008) SARM: a novel Toll-like receptor adaptor, is functionally conserved from arthropod to human. *Molecular immunology* **45**: 1732-1742.
Bertrand G, Coste J, Segarra C, Schved JF, Commes T & Marti J (2004) Use of serial analysis of gene expression (SAGE) technology reveals new granulocytic markers. *Journal of immunological methods* **292**: 43-58.
Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A & Godin I (2005) Three pathways to mature macrophages in the early mouse yolk sac. *Blood* **106**: 3004-3011.
Best JA, Blair DA, Knell J, Yang E, Mayya V, Doedens A, Dustin ML & Goldrath AW (2013) Transcriptional insights into the CD8(+) T cell response to infection and memory T cell formation. *Nat Immunol* **14**: 404-412.

Beutler B (2004) Innate immunity: an overview. *Molecular immunology* **40**: 845-859.
Beyer M, Mallmann MR, Xue J*, et al.* (2012) High-resolution transcriptome of human macrophages. *PloS one* **7**: e45466.
Bhatt DM, Pandya-Jones A, Tong AJ, Barozzi I, Lissner MM, Natoli G, Black DL & Smale ST (2012) Transcript dynamics of proinflammatory genes revealed by sequence analysis of subcellular RNA fractions. *Cell* **150**: 279-290.
Bhattacharyya S, Brown DE, Brewer JA, Vogt SK & Muglia LJ (2007) Macrophage glucocorticoid receptors regulate Toll-like receptor 4–mediated inflammatory responses by selective inhibition of p38 MAP kinase. *Blood* **109**: 4313-4319.
Bhuiyan AA, Li J, Wu Z*, et al.* (2017) Exploring the Genetic Resistance to Gastrointestinal Nematodes Infection in Goat Using RNA-Sequencing. *International Journal of Molecular Sciences* **18**: 751.
Biggs TE, Baker ST, Botham MS, Dhital A, Barton CH & Perry VH (2001) Nramp1 modulates iron homoeostasis in vivo and in vitro: evidence for a role in cellular iron release involving de-acidification of intracellular vesicles. *European journal of immunology* **31**: 2060-2070.
Bishop SC (2015) Genetic resistance to infections in sheep. *Veterinary microbiology* **181**: 2-7.
Biswas SK & Mantovani A (2010) Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature immunology* **11**: 889-896.
Biswas SK & Mantovani A (2012) Orchestration of metabolism by macrophages. *Cell metabolism* **15**: 432-437.
Bittner JJ (1936) SOME POSSIBLE EFFECTS OF NURSING ON THE MAMMARY GLAND TUMOR INCIDENCE IN MICE. *Science (New York, NY)* **84**: 162.
Bochud PY, Hawn TR & Aderem A (2003) Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. *Journal of immunology (Baltimore, Md : 1950)* **170**: 3451-3454.
Bonham KS, Orzalli MH, Hayashi K, Wolf AI, Glanemann C, Weninger W, Iwasaki A, Knipe DM & Kagan JC (2014) A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. *Cell* **156**: 705-716.
Bordon Y (2015) Antiviral immunity. Dual attack by RIG-I. *Nature reviews Immunology* **15**: 70-71.
Borodina T, Adjaye J & Sultan M (2011) A strand-specific library preparation protocol for RNA sequencing. *Methods in enzymology* **500**: 79-98.
Borriello G, Capparelli R, Bianco M, Fenizia D, Alfano F, Capuano F, Ercolini D, Parisi A, Roperto S & Iannelli D (2006) Genetic resistance to Brucella abortus in the water buffalo (Bubalus bubalis). *Infection and immunity* **74**: 2115-2120.
Brandwein SR, Skamene E, Aubut JA, Gervais F & Nesbitt MN (1987) Genetic regulation of lipopolysaccharide-induced interleukin 1 production by murine peritoneal macrophages. *Journal of immunology (Baltimore, Md : 1950)* **138**: 4263-4269.
Braun U, Trösch L, Nydegger F & Hässig M (2013) Evaluation of eating and rumination behaviour in cows using a noseband pressure sensor. *BMC veterinary research* **9**: 164-164.
Bray NL, Pimentel H, Melsted P & Pachter L (2016) Near-optimal probabilistic RNA-seq quantification. *Nature biotechnology* **34**: 525-527.
Brestoff JR & Artis D (2013) Commensal bacteria at the interface of host metabolism and the immune system. *Nature immunology* **14**: 676.
Brodin P & Davis MM (2017) Human immune system variation. *Nature reviews Immunology* **17**: 21-29.
Bronte V & Zanovello P (2005) Regulation of immune responses by L-arginine metabolism. *Nature reviews Immunology* **5**: 641-654.
Broudy VC (1997) Stem cell factor and hematopoiesis. *Blood* **90**: 1345-1364.

Brown GD, Willment JA & Whitehead L (2018) C-type lectins in immunity and homeostasis. *Nature reviews Immunology*.
Brown MS & Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science (New York, NY)* **232**: 34-47.
Broz P & Monack DM (2013) Newly described pattern recognition receptors team up against intracellular pathogens. *Nature Reviews Immunology* **13**: 551.
Bu L, Salto LM, De Leon KJ & De Leon M (2011) Polymorphisms in Fatty Acid Binding Protein 5 Show Association with Type 2 Diabetes. *Diabetes research and clinical practice* **92**: 82-91.
Buchmann K (2014) Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals. *Frontiers in immunology* **5**: 459.
Buckley KM & Rast JP (2012) Dynamic evolution of toll-like receptor multigene families in echinoderms. *Frontiers in immunology* **3**: 136.
Bullard JH, Purdom E, Hansen KD & Dudoit S (2010) Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC bioinformatics* **11**: 94.
Burgess SL, Buonomo E, Carey M, Cowardin C, Naylor C, Noor Z, Wills-Karp M & Petri WA, Jr. (2014) Bone marrow dendritic cells from mice with an altered microbiota provide interleukin 17A-dependent protection against Entamoeba histolytica colitis. *mBio* **5**: e01817.
Burgner D, Jamieson SE & Blackwell JM (2006) Genetic susceptibility to infectious diseases: big is beautiful, but will bigger be even better? *The Lancet Infectious Diseases* **6**: 653-663.
Bush SJ, McCulloch MEB, Summers KM, Hume DA & Clark EL (2017) Integration of quantitated expression estimates from polyA-selected and rRNA-depleted RNA-seq libraries. *BMC Bioinformatics* **18**: 301.
Bush SJ, Muriuki C, McCulloch MEB, Farquhar IL, Clark EL & Hume DA (2018) Cross-species inference of long non-coding RNAs greatly expands the ruminant transcriptome. *Genetics Selection Evolution* **50**: 20.
Cader MZ, Boroviak K, Zhang Q*, et al.* (2016) C13orf31 (FAMIN) is a central regulator of immunometabolic function. *Nature immunology* **17**: 1046-1056.
Cantin AM, Hartl D, Konstan MW & Chmiel JF (2015) Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *Journal of Cystic Fibrosis* **14**: 419-430.
Cao JY & Dixon SJ (2016) Mechanisms of ferroptosis. *Cellular and Molecular Life Sciences* **73**: 2195-2209.
Carninci P & Kasukawa T & Katayama S*, et al.* (2005) The transcriptional landscape of the mammalian genome. *Science (New York, NY)* **309**: 1559-1563.
Carty M, Goodbody R, Schröder M, Stack J, Moynagh PN & Bowie AG (2006) The human adaptor SARM negatively regulates adaptor protein TRIF–dependent Toll-like receptor signaling. *Nature immunology* **7**: 1074.
Castello A, Fischer B, Eichelbaum K*, et al.* (2012) Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* **149**: 1393-1406.
Chamberlain AJ, Vander Jagt CJ, Hayes BJ, Khansefid M, Marett LC, Millen CA, Nguyen TTT & Goddard ME (2015) Extensive variation between tissues in allele specific expression in an outbred mammal. *BMC genomics* **16**: 993.
Chan WK, Lorenzi PL, Anishkin A, Purwaha P, Rogers DM, Sukharev S, Rempe SB & Weinstein JN (2014) The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. *Blood* **123**: 3596-3606.
Chang JT & Nevins JR (2006) GATHER: a systems approach to interpreting genomic signatures. *Bioinformatics (Oxford, England)* **22**: 2926-2933.
Chang JT, Wherry EJ & Goldrath AW (2014) Molecular regulation of effector and memory T cell differentiation. *Nat Immunol* **15**: 1104-1115.

Chang ZL (2010) Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflammation research : official journal of the European Histamine Research Society [et al]* **59**: 791-808.
Chaturvedi R, Asim M, Hoge S*, et al.* (2010) Polyamines Impair Immunity to Helicobacter pylori by Inhibiting L-Arginine Uptake Required for Nitric Oxide Production. *Gastroenterology* **139**: 1686-1698, 1698.e1681-1686.
Chen S-Y, Deng F, Jia X, Li C & Lai S-J (2017) A transcriptome atlas of rabbit revealed by PacBio single-molecule long-read sequencing. *Scientific reports* **7**: 7648.
Chen X, Iliopoulos D, Zhang Q*, et al.* (2014) XBP1 Promotes Triple Negative Breast Cancer By Controlling the HIF1 α Pathway. *Nature* **508**: 103-107.
Chen ZJ (2012) Ubiquitination in signaling to and activation of IKK. *Immunological reviews* **246**: 95-106.
Chessa B, Pereira F, Arnaud F*, et al.* (2009) REVEALING THE HISTORY OF SHEEP DOMESTICATION USING RETROVIRUS INTEGRATIONS. *Science (New York, NY)* **324**: 532-536.
Chhangawala S, Rudy G, Mason CE & Rosenfeld JA (2015) The impact of read length on quantification of differentially expressed genes and splice junction detection. *Genome biology* **16**: 131.
Chitu V & Stanley ER (2006) Colony-stimulating factor-1 in immunity and inflammation. *Current opinion in immunology* **18**: 39-48.
Choudhury RP, Lee JM & Greaves DR (2005) Mechanisms of Disease: macrophage-derived foam cells emerging as therapeutic targets in atherosclerosis. *Nature Clinical Practice Cardiovascular Medicine* **2**: 309.
Clark EL, Bush SJ, McCulloch MEB*, et al.* (2017) A high resolution atlas of gene expression in the domestic sheep (Ovis aries). *PLoS genetics* **13**: e1006997.
Conesa A, Madrigal P, Tarazona S*, et al.* (2016) A survey of best practices for RNA-seq data analysis. *Genome biology* **17**: 13.
Consortium EP (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**: 57-74.
Cooper MD & Alder MN (2006) The Evolution of Adaptive Immune Systems. *Cell* **124**: 815-822.
Cordaux R & Batzer MA (2009) The impact of retrotransposons on human genome evolution. *Nature reviews Genetics* **10**: 691-703.
Costa Rosa LF, Cury Y & Curi R (1991) Hormonal control of macrophage function and glutamine metabolism. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **69**: 309-312.
Costelloe EO, Stacey KJ, Antalis TM & Hume DA (1999) Regulation of the plasminogen activator inhibitor-2 (PAI-2) gene in murine macrophages. Demonstration of a novel pattern of responsiveness to bacterial endotoxin. *Journal of leukocyte biology* **66**: 172-182.
Couillault C, Pujol N, Reboul J, Sabatier L, Guichou JF, Kohara Y & Ewbank JJ (2004) TLR-independent control of innate immunity in Caenorhabditis elegans by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. *Nature immunology* **5**: 488-494.
Crowley JJ, Zhabotynsky V, Sun W*, et al.* (2015) Analyses of allele-specific gene expression in highly divergent mouse crosses identifies pervasive allelic imbalance. *Nature genetics* **47**: 353-360.
Cuadros MA, Coltey P, Carmen Nieto M & Martin C (1992) Demonstration of a phagocytic cell system belonging to the hemopoietic lineage and originating from the yolk sac in the early avian embryo. *Development (Cambridge, England)* **115**: 157-168.
Cui XF, Imaizumi T, Yoshida H, Borden EC & Satoh K (2004) Retinoic acid-inducible gene-I is induced by interferon-gamma and regulates the expression of interferon-gamma

stimulated gene 15 in MCF-7 cells. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **82**: 401-405.
Curi R, de Siqueira Mendes R, de Campos Crispin LA, Norata GD, Sampaio SC & Newsholme P (2017) A past and present overview of macrophage metabolism and functional outcomes. *Clinical science (London, England : 1979)* **131**: 1329-1342.
Daley D, Mani VR, Mohan N*, et al.* (2017) Dectin-1 Activation on Macrophages by Galectin-9 Promotes Pancreatic Carcinoma and Peritumoral Immune-Tolerance. *Nature medicine* **23**: 556-567.
Damiani G, Florio S, Panelli S, Capelli E & Cuccia M (2008) The Bov-A2 retroelement played a crucial role in the evolution of ruminants. *Rivista di biologia* **101**: 375-404.
Danfaer A, Tetens V & Agergaard N (1995) Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. *Comparative biochemistry and physiology Part B, Biochemistry & molecular biology* **111**: 201-210.
Darwin C (1859) *On the origin of species by means of natural selection, or preservation of favoured races in the struggle for life*. London : John Murray, 1859.
Das P, Lahiri A, Lahiri A & Chakravortty D (2010) Modulation of the arginase pathway in the context of microbial pathogenesis: a metabolic enzyme moonlighting as an immune modulator. *PLoS pathogens* **6**: e1000899.
Dávila López M & Samuelsson T (2008) Early evolution of histone mRNA 3' end processing. *RNA* **14**: 1-10.
Davis AS, Vergne I, Master SS, Kyei GB, Chua J & Deretic V (2007) Mechanism of inducible nitric oxide synthase exclusion from mycobacterial phagosomes. *PLoS pathogens* **3**: e186.
de Koning AP, Gu W, Castoe TA, Batzer MA & Pollock DD (2011) Repetitive elements may comprise over two-thirds of the human genome. *PLoS genetics* **7**: e1002384.
de las Casas-Engel M, Dominguez-Soto A, Sierra-Filardi E*, et al.* (2013) Serotonin skews human macrophage polarization through HTR2B and HTR7. *Journal of immunology (Baltimore, Md : 1950)* **190**: 2301-2310.
de Oliveira DC, da Silva Lima F, Sartori T, Santos ACA, Rogero MM & Fock RA (2016) Glutamine metabolism and its effects on immune response: molecular mechanism and gene expression. *Nutrire* **41**: 14.
De Santa F, Narang V, Yap ZH*, et al.* (2009) Jmjd3 contributes to the control of gene expression in LPS-activated macrophages. *The EMBO journal* **28**: 3341-3352.
Desvignes L & Ernst JD (2009) IFNγ-responsive nonhematopoietic cells regulate the immune response to Mycobacterium tuberculosis. *Immunity* **31**: 974-985.
Diatchenko L, Lau YF, Campbell AP*, et al.* (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 6025-6030.
Dimmer KS, Friedrich B, Lang F, Deitmer JW & Broer S (2000) The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. *The Biochemical journal* **350 Pt 1**: 219-227.
Diskin C & Pålsson-McDermott EM (2018) Metabolic Modulation in Macrophage Effector Function. *Frontiers in immunology* **9**.
Dixon SJ & Stockwell BR (2013) The role of iron and reactive oxygen species in cell death. *Nature Chemical Biology* **10**: 9.
Dixon SJ, Lemberg KM, Lamprecht MR*, et al.* (2012) Ferroptosis: An Iron-Dependent Form of Non-Apoptotic Cell Death. *Cell* **149**: 1060-1072.
Dobzhansky T (1973) Nothing in Biology Makes Sense except in the Light of Evolution. *The American Biology Teacher* **35**: 125-129.

Doig TN, Hume DA, Theocharidis T, Goodlad JR, Gregory CD & Freeman TC (2013) Coexpression analysis of large cancer datasets provides insight into the cellular phenotypes of the tumour microenvironment. *BMC genomics* **14**: 469.
Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ & Geczy CL (2013) Functions of S100 proteins. *Current molecular medicine* **13**: 24-57.
Donnelly RP & Finlay DK (2015) Glucose, glycolysis and lymphocyte responses. *Molecular immunology* **68**: 513-519.
Doreau M & Ferlay A (1994) Digestion and utilisation of fatty acids by ruminants. *Animal Feed Science and Technology* **45**: 379-396.
Dougherty RW (1968) Eructation in ruminants. *Annals of the New York Academy of Sciences* **150**: 22-26.
Draghici S, Khatri P, Eklund AC & Szallasi Z (2006) Reliability and reproducibility issues in DNA microarray measurements. *Trends in genetics : TIG* **22**: 101-109.
Ehrlich P (1900) Croonian lecture.—On immunity with special reference to cell life. *Proceedings of the Royal Society of London* **66**: 424-448.
Ekblom R & Wolf JBW (2014) A field guide to whole-genome sequencing, assembly and annotation. *Evolutionary Applications* **7**: 1026-1042.
El Kasmi KC & Stenmark KR (2015) Contribution of metabolic reprogramming to macrophage plasticity and function. *Seminars in immunology* **27**: 267-275.
Elsik CG & Tellam RL & Worley KC*, et al.* (2009) The genome sequence of taurine cattle: a window to ruminant biology and evolution. *Science (New York, NY)* **324**: 522-528.
Enright AJ, Van Dongen S & Ouzounis CA (2002) An efficient algorithm for large-scale detection of protein families. *Nucleic acids research* **30**: 1575-1584.
Entrican G, Wattegedera S, Rocchi M & Wheelhouse N (2009) Pregnancy, indoleamine 2,3-dioxygenase (IDO) and chlamydial abortion: an unresolved paradox. *Veterinary microbiology* **135**: 98-102.
Ernst J, Kheradpour P, Mikkelsen TS*, et al.* (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* **473**: 43-49.
Erny D, Hrabe de Angelis AL, Jaitin D*, et al.* (2015) Host microbiota constantly control maturation and function of microglia in the CNS. *Nature neuroscience* **18**: 965-977.
Esteban-Redondo I, Maley SW, Thomson K, Nicoll S, Wright S, Buxton D & Innes EA (1999) Detection of T. gondii in tissues of sheep and cattle following oral infection. *Veterinary parasitology* **86**: 155-171.
Eyles JL, Roberts AW, Metcalf D & Wicks IP (2006) Granulocyte colony-stimulating factor and neutrophils--forgotten mediators of inflammatory disease. *Nature clinical practice Rheumatology* **2**: 500-510.
Fagerholm SC, Varis M, Stefanidakis M, Hilden TJ & Gahmberg CG (2006) alpha-Chain phosphorylation of the human leukocyte CD11b/CD18 (Mac-1) integrin is pivotal for integrin activation to bind ICAMs and leukocyte extravasation. *Blood* **108**: 3379-3386.
Fairbairn L, Kapetanovic R, Sester DP & Hume DA (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. *Journal of leukocyte biology* **89**: 855-871.
Fairbairn L, Kapetanovic R, Beraldi D, Sester DP, Tuggle CK, Archibald AL & Hume DA (2013) Comparative analysis of monocyte subsets in the pig. *Journal of immunology (Baltimore, Md : 1950)* **190**: 6389-6396.
Fairfax BP, Humburg P, Makino S*, et al.* (2014) Innate immune activity conditions the effect of regulatory variants upon monocyte gene expression. *Science (New York, NY)* **343**: 1246949.
Fang Y & Fullwood MJ (2016) Roles, Functions, and Mechanisms of Long Non-coding RNAs in Cancer. *Genomics, Proteomics & Bioinformatics* **14**: 42-54.

Fanning LJ (2011) Retroviruses: Molecular Biology, Genomics and Pathogenesis. *Clinical Infectious Diseases* **52**: 280-280.
Fatovich DM & Phillips M (2017) The probability of probability and research truths. *Emerg Med Australas*.
Faulkner GJ, Kimura Y, Daub CO*, et al.* (2009) The regulated retrotransposon transcriptome of mammalian cells. *Nature genetics* **41**: 563-571.
Fazzari P, Horre K, Arranz AM, Frigerio CS, Saito T, Saido TC & De Strooper B (2017) PLD3 gene and processing of APP. *Nature* **541**: E1.
Feingold KR, Shigenaga JK, Kazemi MR, McDonald CM, Patzek SM, Cross AS, Moser A & Grunfeld C (2012) Mechanisms of triglyceride accumulation in activated macrophages. *Journal of leukocyte biology* **92**: 829-839.
Feng H & Stockwell BR (2018) Unsolved mysteries: How does lipid peroxidation cause ferroptosis? *PLOS Biology* **16**: e2006203.
Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM & Golenbock DT (2003) LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *The Journal of experimental medicine* **198**: 1043-1055.
Flint HJ, Bayer EA, Rincon MT, Lamed R & White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nature reviews Microbiology* **6**: 121-131.
Foell D, Frosch M, Sorg C & Roth J (2004) Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clinica Chimica Acta* **344**: 37-51.
Fogel LA, Sun MM, Geurs TL, Carayannopoulos LN & French AR (2013) Markers of nonselective and specific NK cell activation. *J Immunol* **190**: 6269-6276.
Forbes JR & Gros P (2001) Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends in microbiology* **9**: 397-403.
Forbes JR & Gros P (2003) Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane. *Blood* **102**: 1884-1892.
Foster SL, Hargreaves DC & Medzhitov R (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447**: 972-978.
Franca LM, Freitas LN, Chagas VT, Coelho CF, Barroso WA, Costa GC, Silva LA, Debbas V, Laurindo FR & Paes AM (2014) Mechanisms underlying hypertriglyceridemia in rats with monosodium L-glutamate-induced obesity: evidence of XBP-1/PDI/MTP axis activation. *Biochemical and biophysical research communications* **443**: 725-730.
Franchi L, Warner N, Viani K & Nuñez G (2009) Function of Nod-like Receptors in Microbial Recognition and Host Defense. *Immunological reviews* **227**: 106-128.
Franchi L, Park JH, Shaw MH, Marina-Garcia N, Chen G, Kim YG & Nunez G (2008) Intracellular NOD-like receptors in innate immunity, infection and disease. *Cellular microbiology* **10**: 1-8.
Frazee AC, Pertea G, Jaffe AE, Langmead B, Salzberg SL & Leek JT (2015) Ballgown bridges the gap between transcriptome assembly and expression analysis. *Nature biotechnology* **33**: 243-246.
Freeman T, Ivens A, Baillie JK, Beraldi D, Barnett M, Dorward D, Downing A, Fairbairn L, Kapetanovic R & Raza S (2012) A gene expression atlas of the domestic pig. *BMC biology* **10**: 90.
Freeman TC, Ivens A, Baillie JK*, et al.* (2012) A gene expression atlas of the domestic pig. *BMC biology* **10**: 90.
Freemerman AJ, Johnson AR, Sacks GN, Milner JJ, Kirk EL, Troester MA, Macintyre AN, Goraksha-Hicks P, Rathmell JC & Makowski L (2014) Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *The Journal of biological chemistry* **289**: 7884-7896.

Fritsche G, Nairz M, Theurl I, Mair S, Bellmann-Weiler R, Barton HC & Weiss G (2007) Modulation of macrophage iron transport by Nramp1 (Slc11a1). *Immunobiology* **212**: 751-757.
Frolov A, Zielinski SE, Crowley JR, Dudley-Rucker N, Schaffer JE & Ory DS (2003) NPC1 and NPC2 regulate cellular cholesterol homeostasis through generation of low density lipoprotein cholesterol-derived oxysterols. *The Journal of biological chemistry* **278**: 25517-25525.
Frolov AE, Godwin AK & Favorova OO (2003) Differential Gene Expression Analysis by DNA Microarray Technology and Its Application in Molecular Oncology. *Molecular Biology* **37**: 486-494.
Fu J FA, Collado-Torres L, Jaffe AE and Leek JT (2016) ballgown: Flexible, isoform-level differential expression analysis. R package version 2.6.0. p.^pp.
Fukuzumi M, Shinomiya H, Shimizu Y, Ohishi K & Utsumi S (1996) Endotoxin-induced enhancement of glucose influx into murine peritoneal macrophages via GLUT1. *Infection and immunity* **64**: 108-112.
Gaber T, Strehl C & Buttgereit F (2017) Metabolic regulation of inflammation. *Nature reviews Rheumatology* **13**: 267-279.
Garceau V, Smith J, Paton IR, Davey M, Fares MA, Sester DP, Burt DW & Hume DA (2010) Pivotal Advance: Avian colony-stimulating factor 1 (CSF-1), interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. *Journal of leukocyte biology* **87**: 753-764.
Gautier EL, Shay T, Miller J*, et al.* (2012) Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nature immunology* **13**: 1118-1128.
Gautier EL, Shay T, Miller J*, et al.* (2012) Gene expression profiles and transcriptional regulatory pathways underlying mouse tissue macrophage identity and diversity. *Nature immunology* **13**: 1118-1128.
Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M & Ley K (2010) Development of monocytes, macrophages, and dendritic cells. *Science (New York, NY)* **327**: 656-661.
Geng H & Tan X-D (2016) Functional diversity of long non-coding RNAs in immune regulation. *Genes & Diseases* **3**: 72-81.
Gerstein MB, Kundaje A, Hariharan M*, et al.* (2012) Architecture of the human regulatory network derived from ENCODE data. *Nature* **489**: 91-100.
Ghisletti S, Barozzi I, Mietton F*, et al.* (2010) Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity* **32**: 317-328.
Gibbings SL, Goyal R, Desch AN, Leach SM, Prabagar M, Atif SM, Bratton DL, Janssen W & Jakubzick CV (2015) Transcriptome analysis highlights the conserved difference between embryonic and postnatal-derived alveolar macrophages. *Blood* **126**: 1357-1366.
Gifford R & Tristem M (2003) The Evolution, Distribution and Diversity of Endogenous Retroviruses. *Virus Genes* **26**: 291-315.
Gilchrist M, Henderson WR, Jr., Clark AE, Simmons RM, Ye X, Smith KD & Aderem A (2008) Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation. *The Journal of experimental medicine* **205**: 2349-2357.
Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Roach JC, Kennedy K, Hai T, Bolouri H & Aderem A (2006) Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* **441**: 173-178.
Ginhoux F & Jung S (2014) Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature reviews Immunology* **14**: 392-404.
Ginhoux F & Guilliams M (2016) Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* **44**: 439-449.

Ginhoux F, Liu K, Helft J*, et al.* (2009) The origin and development of nonlymphoid tissue CD103+ DCs. *The Journal of experimental medicine* **206**: 3115-3130.
Glendinning L, Wright S, Pollock J, Tennant P, Collie D & McLachlan G (2016) Variability of the Sheep Lung Microbiota. *Applied and Environmental Microbiology* **82**: 3225-3238.
Godfroy JI, Roostan M, Moroz YS, Korendovych IV & Yin H (2012) Isolated Toll-like Receptor Transmembrane Domains Are Capable of Oligomerization. *PloS one* **7**: e48875.
Gordon S (2002) Pattern Recognition Receptors. *Cell* **111**: 927-930.
Gordon S (2016) Elie Metchnikoff, the Man and the Myth. *Journal of innate immunity* **8**: 223-227.
Gordon S & Martinez FO (2010) Alternative activation of macrophages: mechanism and functions. *Immunity* **32**: 593-604.
Gordon S & Pluddemann A (2017) Tissue macrophages: heterogeneity and functions. *BMC biology* **15**: 53.
Gordon S, Pluddemann A & Martinez Estrada F (2014) Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunological reviews* **262**: 36-55.
Goyette J, Yan WX, Yamen E*, et al.* (2009) Pleiotropic roles of S100A12 in coronary atherosclerotic plaque formation and rupture. *Journal of immunology (Baltimore, Md : 1950)* **183**: 593-603.
Goytain A, Hines RM & Quamme GA (2008) Functional characterization of NIPA2, a selective Mg2+ transporter. *American journal of physiology Cell physiology* **295**: C944-953.
Grabmuller M, Madea B & Courts C (2015) Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. *Forensic science international Genetics* **16**: 195-202.
Graur D (2017) An Upper Limit on the Functional Fraction of the Human Genome. *Genome biology and evolution* **9**: 1880-1885.
Grieves JL, Dick EJ, Jr., Schlabritz-Loutsevich NE, Butler SD, Leland MM, Price SE, Schmidt CR, Nathanielsz PW & Hubbard GB (2008) Barbiturate euthanasia solution-induced tissue artifact in nonhuman primates. *Journal of medical primatology* **37**: 154-161.
Groh L, Keating ST, Joosten LAB, Netea MG & Riksen NP (2017) Monocyte and macrophage immunometabolism in atherosclerosis. *Seminars in immunopathology*.
Gross L (1951) "Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK-leukemic extracts, or AK-embrvos. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY)* **76**: 27-32.
Gross SR, Sin CG, Barraclough R & Rudland PS (2014) Joining S100 proteins and migration: for better or for worse, in sickness and in health. *Cellular and molecular life sciences : CMLS* **71**: 1551-1579.
Gruenheid S, Pinner E, Desjardins M & Gros P (1997) Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *The Journal of experimental medicine* **185**: 717-730.
Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, Segura E, Tussiwand R & Yona S (2014) Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature reviews Immunology* **14**: 571-578.
Guilliams M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, Deswarte K, Malissen B, Hammad H & Lambrecht BN (2013) Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *The Journal of experimental medicine* **210**: 1977-1992.
Hacker H, Tseng PH & Karin M (2011) Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nature reviews Immunology* **11**: 457-468.

Hackmann TJ & Spain JN (2010) Invited review: ruminant ecology and evolution: perspectives useful to ruminant livestock research and production. *J Dairy Sci* **93**: 1320-1334.
Hagenbuchner J, Kiechl-Kohlendorfer U, Obexer P & Ausserlechner MJ (2016) BIRC5/Survivin as a target for glycolysis inhibition in high-stage neuroblastoma. *Oncogene* **35**: 2052-2061.
Hamann L, Kumpf O, Muller M, Visintin A, Eckert J, Schlag PM & Schumann RR (2004) A coding mutation within the first exon of the human MD-2 gene results in decreased lipopolysaccharide-induced signaling. *Genes and immunity* **5**: 283-288.
Hamilton JA (2008) Colony-stimulating factors in inflammation and autoimmunity. *Nature reviews Immunology* **8**: 533-544.
Hamilton JA, Cook AD & Tak PP (2016) Anti-colony-stimulating factor therapies for inflammatory and autoimmune diseases. *Nature reviews Drug discovery* **16**: 53-70.
Hamza I & Dailey HA (2012) One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans. *Biochimica et biophysica acta* **1823**: 1617-1632.
Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK & Kjems J (2013) Natural RNA circles function as efficient microRNA sponges. *Nature* **495**: 384-388.
Hara T, Kimura I, Inoue D, Ichimura A & Hirasawa A (2013) Free fatty acid receptors and their role in regulation of energy metabolism. *Reviews of physiology, biochemistry and pharmacology* **164**: 77-116.
Hargreaves DC, Horng T & Medzhitov R (2009) Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell* **138**: 129-145.
Hashimoto D, Chow A, Noizat C*, et al.* (2013) Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* **38**: 792-804.
Haziot A, Ferrero E, Lin XY, Stewart CL & Goyert SM (1995) CD14-deficient mice are exquisitely insensitive to the effects of LPS. *Progress in clinical and biological research* **392**: 349-351.
He L, Weber KJ & Schilling JD (2016) Glutamine Modulates Macrophage Lipotoxicity. *Nutrients* **8**: 215.
He W, Miao FJ, Lin DC, Schwandner RT, Wang Z, Gao J, Chen JL, Tian H & Ling L (2004) Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature* **429**: 188-193.
Heby O, Persson L & Rentala M (2007) Targeting the polyamine biosynthetic enzymes: a promising approach to therapy of African sleeping sickness, Chagas' disease, and leishmaniasis. *Amino acids* **33**: 359-366.
Hedrick SM, Nielsen EA, Kavaler J, Cohen DI & Davis MM (1984) Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature* **308**: 153-158.
Heinz S, Haehnel V, Karaghiosoff M, Schwarzfischer L, Muller M, Krause SW & Rehli M (2003) Species-specific regulation of Toll-like receptor 3 genes in men and mice. *The Journal of biological chemistry* **278**: 21502-21509.
Heinz S, Romanoski CE, Benner C, Allison KA, Kaikkonen MU, Orozco LD & Glass CK (2013) Effect of natural genetic variation on enhancer selection and function. *Nature* **503**: 487-492.
Hemmi H & Akira S (2005) TLR signalling and the function of dendritic cells. *Chemical immunology and allergy* **86**: 120-135.
Heng TS & Painter MW (2008) The Immunological Genome Project: networks of gene expression in immune cells. *Nature immunology* **9**: 1091-1094.
Hofacre A & Fan H (2010) Jaagsiekte Sheep Retrovirus Biology and Oncogenesis. *Viruses* **2**: 2618-2648.
Hoffmann JA (2003) The immune response of Drosophila. *Nature* **426**: 33-38.

Hoffmann R & Valencia A (2004) A gene network for navigating the literature. *Nature genetics* **36**: 664.
Hofmann Bowman MA, Gawdzik J, Bukhari U, Husain AN, Toth PT, Kim G, Earley J & McNally EM (2011) S100A12 in Vascular Smooth Muscle Accelerates Vascular Calcification in Apolipoprotein E–Null Mice by Activating an Osteogenic Gene Regulatory Program. *Arteriosclerosis, thrombosis, and vascular biology* **31**: 337-344.
Horng T, Barton GM, Flavell RA & Medzhitov R (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420**: 329-333.
Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K & Akira S (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *Journal of immunology (Baltimore, Md : 1950)* **162**: 3749-3752.
Hoshino S, Sarumaru K & Morimoto K (1966) Ammonia Anabolism in Ruminants. *Journal of Dairy Science* **49**: 1523-1528.
Hotamisligil GS (2017) Foundations of Immunometabolism and Implications for Metabolic Health and Disease. *Immunity* **47**: 406-420.
Hultmark D (1994) Macrophage differentiation marker MyD88 is a member of the Toll/IL-1 receptor family. *Biochemical and biophysical research communications* **199**: 144-146.
Hume DA (2006) The mononuclear phagocyte system. *Current opinion in immunology* **18**: 49-53.
Hume DA (2008) Differentiation and heterogeneity in the mononuclear phagocyte system. *Mucosal immunology* **1**: 432-441.
Hume DA (2015) The Many Alternative Faces of Macrophage Activation. *Frontiers in immunology* **6**: 370.
Hume DA & Freeman TC (2014) Transcriptomic analysis of mononuclear phagocyte differentiation and activation. *Immunological reviews* **262**: 74-84.
Hume DA, Perry VH & Gordon S (1984) The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80: macrophages associated with epithelia. *The Anatomical record* **210**: 503-512.
Hume DA, Summers KM & Rehli M (2016) Transcriptional Regulation and Macrophage Differentiation. *Microbiology spectrum* **4**.
Hume DA, Mabbott N, Raza S & Freeman TC (2013) Can DCs be distinguished from macrophages by molecular signatures? *Nature immunology* **14**: 187-189.
Hume DA, Wollscheid-Lengeling E, Rojo R & Pridans C (2017) The evolution of the macrophage-specific enhancer (Fms intronic regulatory element) within the CSF1R locus of vertebrates. *Scientific reports* **7**: 17115.
Hume DA, Underhill DM, Sweet MJ, Ozinsky AO, Liew FY & Aderem A (2001) Macrophages exposed continuously to lipopolysaccharide and other agonists that act via toll-like receptors exhibit a sustained and additive activation state. *BMC immunology* **2**: 11.
Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA & Ravasi T (2002) The mononuclear phagocyte system revisited. *Journal of leukocyte biology* **72**: 621-627.
Hussell T & Bell TJ (2014) Alveolar macrophages: plasticity in a tissue-specific context. *Nature Reviews Immunology* **14**: 81.
Ichikawa Y, Pluznik DH & Sachs L (1966) In vitro control of the development of macrophage and granulocyte colonies. *Proceedings of the National Academy of Sciences of the United States of America* **56**: 488-495.
Iles KE & Forman HJ (2002) Macrophage signaling and respiratory burst. *Immunologic research* **26**: 95-105.

Imaizumi T, Aratani S, Nakajima T*, et al.* (2002) Retinoic acid-inducible gene-I is induced in endothelial cells by LPS and regulates expression of COX-2. *Biochemical and biophysical research communications* **292**: 274-279.
Infantino V, Iacobazzi V, Menga A, Avantaggiati ML & Palmieri F (2014) A key role of the mitochondrial citrate carrier (SLC25A1) in TNFalpha- and IFNgamma-triggered inflammation. *Biochimica et biophysica acta* **1839**: 1217-1225.
Irvine KM, Andrews MR, Fernandez-Rojo MA, Schroder K, Burns CJ, Su S, Wilks AF, Parton RG, Hume DA & Sweet MJ (2009) Colony-stimulating factor-1 (CSF-1) delivers a proatherogenic signal to human macrophages. *Journal of leukocyte biology* **85**: 278-288.
Isaacs A & Lindenmann J (1987) Virus interference. I. The interferon. By A. Isaacs and J. Lindenmann, 1957. *Journal of interferon research* **7**: 429-438.
Janeway CA, Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor symposia on quantitative biology* **54 Pt 1**: 1-13.
Janeway CA, Jr. & Medzhitov R (2002) Innate immune recognition. *Annual review of immunology* **20**: 197-216.
Jang MH, Herber DM, Jiang X, Nandi S, Dai XM, Zeller G, Stanley ER & Kelley VR (2006) Distinct in vivo roles of colony-stimulating factor-1 isoforms in renal inflammation. *Journal of immunology (Baltimore, Md : 1950)* **177**: 4055-4063.
Jenkins SJ & Hume DA (2014) Homeostasis in the mononuclear phagocyte system. *Trends in immunology* **35**: 358-367.
Jenkins SJ, Ruckerl D, Thomas GD, Hewitson JP, Duncan S, Brombacher F, Maizels RM, Hume DA & Allen JE (2013) IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *The Journal of experimental medicine* **210**: 2477-2491.
Jensen RA (2001) Orthologs and paralogs - we need to get it right. *Genome biology* **2**: interactions1002.1001-interactions1002.1003.
Jenssen TK, Laegreid A, Komorowski J & Hovig E (2001) A literature network of human genes for high-throughput analysis of gene expression. *Nature genetics* **28**: 21-28.
Jha Abhishek K, Huang Stanley C-C, Sergushichev A*, et al.* (2015) Network Integration of Parallel Metabolic and Transcriptional Data Reveals Metabolic Modules that Regulate Macrophage Polarization. *Immunity* **42**: 419-430.
Jiang H, Shi H, Sun M*, et al.* (2016) PFKFB3-Driven Macrophage Glycolytic Metabolism Is a Crucial Component of Innate Antiviral Defense. *Journal of immunology (Baltimore, Md : 1950)* **197**: 2880-2890.
Jiang Q, Akashi S, Miyake K & Petty HR (2000) Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. *Journal of immunology (Baltimore, Md : 1950)* **165**: 3541-3544.
Jiang Y, Xie M, Chen W*, et al.* (2014) The sheep genome illuminates biology of the rumen and lipid metabolism. *Science (New York, NY)* **344**: 1168-1173.
Jubb AW, Young RS, Hume DA & Bickmore WA (2016) Enhancer Turnover Is Associated with a Divergent Transcriptional Response to Glucocorticoid in Mouse and Human Macrophages. *Journal of immunology (Baltimore, Md : 1950)* **196**: 813-822.
Kagan JC & Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* **125**: 943-955.
Kaikkonen MU, Spann NJ, Heinz S*, et al.* (2013) Remodeling of the enhancer landscape during macrophage activation is coupled to enhancer transcription. *Molecular cell* **51**: 310-325.
Kaminski DA, Wei C, Qian Y, Rosenberg AF & Sanz I (2012) Advances in human B cell phenotypic profiling. *Frontiers in immunology* **3**: 302.

Kane M, Zang TM, Rihn SJ, Zhang F, Kueck T, Alim M, Schoggins J, Rice CM, Wilson SJ & Bieniasz PD (2016) Identification of Interferon-Stimulated Genes with Antiretroviral Activity. *Cell host & microbe* **20**: 392-405.
Kang JX, Bell J, Leaf A, Beard RL & Chandraratna RAS (1998) Retinoic acid alters the intracellular trafficking of the mannose-6-phosphate/insulin-like growth factor II receptor and lysosomal enzymes. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 13687-13691.
Kapetanovic R, Fairbairn L, Beraldi D, Sester DP, Archibald AL, Tuggle CK & Hume DA (2012) Pig bone marrow-derived macrophages resemble human macrophages in their response to bacterial lipopolysaccharide. *Journal of immunology (Baltimore, Md : 1950)* **188**: 3382-3394.
Kapetanovic R, Fairbairn L, Beraldi D, Sester DP, Archibald AL, Tuggle CK & Hume DA (2012) Pig bone marrow-derived macrophages resemble human macrophages in their response to bacterial lipopolysaccharide. *The Journal of Immunology* **188**: 3382-3394.
Kapetanovic R, Fairbairn L, Downing A, Beraldi D, Sester DP, Freeman TC, Tuggle CK, Archibald AL & Hume DA (2013) The impact of breed and tissue compartment on the response of pig macrophages to lipopolysaccharide. *BMC genomics* **14**: 581.
Karagianni AE, Kapetanovic R, Summers KM, McGorum BC, Hume DA & Pirie RS (2016) Comparative transcriptome analysis of equine alveolar macrophages. *Equine Vet J*.
Kaser A, Lee A-H, Franke A*, et al.* (2008) XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* **134**: 743-756.
Katz Y, Wang ET, Silterra J, Schwartz S, Wong B, Thorvaldsdottir H, Robinson JT, Mesirov JP, Airoldi EM & Burge CB (2015) Quantitative visualization of alternative exon expression from RNA-seq data. *Bioinformatics (Oxford, England)* **31**: 2400-2402.
Kaufmann SH, Cole ST, Mizrahi V, Rubin E & Nathan C (2005) Mycobacterium tuberculosis and the host response. *The Journal of experimental medicine* **201**: 1693-1697.
Kawahara K, Hohjoh H, Inazumi T, Tsuchiya S & Sugimoto Y (2015) Prostaglandin E2-induced inflammation: Relevance of prostaglandin E receptors. *Biochimica et biophysica acta* **1851**: 414-421.
Kawai T & Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* **11**: 373-384.
Kawai T & Akira S (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**: 637-650.
Kawaji H, Kasukawa T, Forrest A, Carninci P & Hayashizaki Y (2017) The FANTOM5 collection, a data series underpinning mammalian transcriptome atlases in diverse cell types. *Scientific data* **4**: 170113.
Kawasaki T & Kawai T (2014) Toll-Like Receptor Signaling Pathways. *Frontiers in immunology* **5**: 461.
Kay RN (1966) The influence of saliva on digestion in ruminants. *World review of nutrition and dietetics* **6**: 292-325.
Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A & Maier B (2009) A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci U S A* **106**: 21407-21412.
Kellis M, Wold B, Snyder MP*, et al.* (2014) Defining functional DNA elements in the human genome. *Proceedings of the National Academy of Sciences of the United States of America* **111**: 6131-6138.
Kelly B & O'Neill LA (2015) Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell research* **25**: 771-784.
Kennedy DG, Young PB, McCaughey WJ, Kennedy S & Blanchflower WJ (1991) Rumen succinate production may ameliorate the effects of cobalt-vitamin B-12 deficiency on methylmalonyl CoA mutase in sheep. *The Journal of nutrition* **121**: 1236-1242.

Khadom NJ, Dedieu JF & Viso M (1985) Bovine alveolar macrophage: a review. *Annales de recherches veterinaires Annals of veterinary research* **16**: 175-183.
Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J & Schwartz DA (2002) Toll-like receptor 4 polymorphisms and atherogenesis. *The New England journal of medicine* **347**: 185-192.
Kikuchi K & Kondo M (2006) Developmental switch of mouse hematopoietic stem cells from fetal to adult type occurs in bone marrow after birth. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 17852-17857.
Kim DH & Ausubel FM (2005) Evolutionary perspectives on innate immunity from the study of Caenorhabditis elegans. *Current opinion in immunology* **17**: 4-10.
Kim H (2017) The transcription factor MafB promotes anti-inflammatory M2 polarization and cholesterol efflux in macrophages. *Scientific reports* **7**: 7591.
Kim SY, Jeong J-M, Kim SJ*, et al.* (2017) Pro-inflammatory hepatic macrophages generate ROS through NADPH oxidase 2 via endocytosis of monomeric TLR4–MD2 complex. *Nature communications* **8**: 2247.
Kimbrell DA & Beutler B (2001) The evolution and genetics of innate immunity. *Nature Reviews Genetics* **2**: 256.
Kishino A, Hayashi K, Hidai C, Masuda T, Nomura Y & Oshima T (2017) XBP1-FoxO1 interaction regulates ER stress-induced autophagy in auditory cells. *Scientific reports* **7**: 4442.
Kleingardner JG & Bren KL (2015) Biological significance and applications of heme c proteins and peptides. *Accounts of chemical research* **48**: 1845-1852.
Kleinjan DA & van Heyningen V (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *American journal of human genetics* **76**: 8-32.
Klemm D, Heublein B, Fink HP & Bohn A (2005) Cellulose: fascinating biopolymer and sustainable raw material. *Angewandte Chemie (International ed in English)* **44**: 3358-3393.
Knight JC (2004) Allele-specific gene expression uncovered. *Trends in Genetics* **20**: 113-116.
Knutson M & Wessling-Resnick M (2003) Iron metabolism in the reticuloendothelial system. *Critical reviews in biochemistry and molecular biology* **38**: 61-88.
Koblansky AA, Jankovic D, Oh H, Hieny S, Sungnak W, Mathur R, Hayden MS, Akira S, Sher A & Ghosh S (2013) Recognition of Profilin by Toll-like Receptor 12 Is Critical for Host Resistance to Toxoplasma gondii. *Immunity* **38**: 119-130.
Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, Li S, Wesche H & Martin MU (2004) Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *The Journal of biological chemistry* **279**: 5227-5236.
Kondo T, Kawai T & Akira S (2012) Dissecting negative regulation of Toll-like receptor signaling. *Trends in immunology* **33**: 449-458.
Kopf M, Schneider C & Nobs SP (2014) The development and function of lung-resident macrophages and dendritic cells. *Nature immunology* **16**: 36.
Kornfeld S (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annual review of biochemistry* **61**: 307-330.
Korolnek T & Hamza I (2015) Macrophages and iron trafficking at the birth and death of red cells. *Blood* **125**: 2893-2897.
Kour S, Garimella MG, Shiroor DA*, et al.* (2016) IL-3 Decreases Cartilage Degeneration by Downregulating Matrix Metalloproteinases and Reduces Joint Destruction in Osteoarthritic Mice. *Journal of immunology (Baltimore, Md : 1950)* **196**: 5024-5035.
Kozarewa I, Ning Z, Quail MA, Sanders MJ, Berriman M & Turner DJ (2009) Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. *Nat Methods* **6**: 291-295.

Krall AS, Xu S, Graeber TG, Braas D & Christofk HR (2016) Asparagine promotes cancer cell proliferation through use as an amino acid exchange factor. *Nature communications* **7**: 11457.
Krause DO & Russell JB (1996) How Many Ruminal Bacteria Are There? *Journal of Dairy Science* **79**: 1467-1475.
Krebs DL & Hilton DJ (2001) SOCS proteins: negative regulators of cytokine signaling. *Stem cells (Dayton, Ohio)* **19**: 378-387.
Krupp M, Marquardt JU, Sahin U, Galle PR, Castle J & Teufel A (2012) RNA-Seq Atlas--a reference database for gene expression profiling in normal tissue by next-generation sequencing. *Bioinformatics (Oxford, England)* **28**: 1184-1185.
Kugelberg E (2014) Glucocorticoids shift response in macrophages. *Nature Reviews Immunology* **14**: 66.
Kukurba KR & Montgomery SB (2015) RNA Sequencing and Analysis. *Cold Spring Harbor protocols* **2015**: 951-969.
Kumar S & Baglioni C (1991) Protection from tumor necrosis factor-mediated cytolysis by overexpression of plasminogen activator inhibitor type-2. *The Journal of biological chemistry* **266**: 20960-20964.
Kumar S, Ambrosini G & Bucher P (2017) SNP2TFBS – a database of regulatory SNPs affecting predicted transcription factor binding site affinity. *Nucleic acids research* **45**: D139-D144.
Kwakkenbos MJ, Kop EN, Stacey M, Matmati M, Gordon S, Lin H-H & Hamann J (2004) The EGF-TM7 family: a postgenomic view. *Immunogenetics* **55**: 655-666.
Langmead B & Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature methods* **9**: 357-359.
Langston PK, Shibata M & Horng T (2017) Metabolism Supports Macrophage Activation. *Frontiers in immunology* **8**: 61.
Lantz CS, Boesiger J, Song CH, Mach N, Kobayashi T, Mulligan RC, Nawa Y, Dranoff G & Galli SJ (1998) Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature* **392**: 90-93.
Larson G, Piperno DR, Allaby RG*, et al.* (2014) Current perspectives and the future of domestication studies. *Proceedings of the National Academy of Sciences of the United States of America* **111**: 6139-6146.
Latz E, Xiao TS & Stutz A (2013) Activation and regulation of the inflammasomes. *Nature Reviews Immunology* **13**: 397.
Lawrence T & Natoli G (2011) Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nature reviews Immunology* **11**: 750-761.
Leavy O (2007) XBP1 — aiding survival. *Nature Reviews Immunology* **7**: 836.
Lee BL, Moon JE, Shu JH, Yuan L, Newman ZR, Schekman R & Barton GM (2013) UNC93B1 mediates differential trafficking of endosomal TLRs. *eLife* **2**: e00291.
Lemaitre B, Nicolas E, Michaut L, Reichhart JM & Hoffmann JA (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**: 973-983.
Li D & Yang MQ (2017) Identification and characterization of conserved lncRNAs in human and rat brain. *BMC Bioinformatics* **18**: 489.
Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G & Durbin R (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)* **25**: 2078-2079.
Li W, Cowley A, Uludag M, Gur T, McWilliam H, Squizzato S, Park YM, Buso N & Lopez R (2015) The EMBL-EBI bioinformatics web and programmatic tools framework. *Nucleic acids research* **43**: W580-584.

Liang P & Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science (New York, NY)* **257**: 967-971.
Lighten J, Papadopulos AST, Mohammed RS, Ward BJ, G. Paterson I, Baillie L, Bradbury IR, Hendry AP, Bentzen P & van Oosterhout C (2017) Evolutionary genetics of immunological supertypes reveals two faces of the Red Queen. *Nature communications* **8**: 1294.
Lin H, Lee E, Hestir K*, et al.* (2008) Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science (New York, NY)* **320**: 807-811.
Lin S-C, Lo Y-C & Wu H (2010) Helical assembly in the MyD88:IRAK4:IRAK2 complex in TLR/IL-1R signaling. *Nature* **465**: 885-890.
Lipshutz RJ, Fodor SP, Gingeras TR & Lockhart DJ (1999) High density synthetic oligonucleotide arrays. *Nature genetics* **21**: 20-24.
Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH & Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell* **133**: 523-536.
Litman GW, Rast JP & Fugmann SD (2010) The origins of vertebrate adaptive immunity. *Nature Reviews Immunology* **10**: 543.
Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, Chu FF, Randolph GJ, Rudensky AY & Nussenzweig M (2009) In vivo analysis of dendritic cell development and homeostasis. *Science (New York, NY)* **324**: 392-397.
Liu Y, Yang L, An H, Chang Y, Zhang W, Zhu Y, Xu L & Xu J (2015) High expression of Solute Carrier Family 1, member 5 (SLC1A5) is associated with poor prognosis in clear-cell renal cell carcinoma. *Scientific reports* **5**: 16954.
Lizio M, Harshbarger J, Shimoji H*, et al.* (2015) Gateways to the FANTOM5 promoter level mammalian expression atlas. *Genome biology* **16**: 22.
Lord KA, Hoffman-Liebermann B & Liebermann DA (1990) Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. *Oncogene* **5**: 1095-1097.
Lorenz E, Mira JP, Frees KL & Schwartz DA (2002) Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Archives of internal medicine* **162**: 1028-1032.
Lorenz E, Hallman M, Marttila R, Haataja R & Schwartz DA (2002) Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population. *Pediatric research* **52**: 373-376.
Lorenz E, Muhlebach MS, Tessier PA, Alexis NE, Duncan Hite R, Seeds MC, Peden DB & Meredith W (2008) Different expression ratio of S100A8/A9 and S100A12 in acute and chronic lung diseases. *Respiratory medicine* **102**: 567-573.
Lu C & King RD (2009) An investigation into the population abundance distribution of mRNAs, proteins, and metabolites in biological systems. *Bioinformatics (Oxford, England)* **25**: 2020-2027.
Lu YC, Yeh WC & Ohashi PS (2008) LPS/TLR4 signal transduction pathway. *Cytokine* **42**: 145-151.
Lux CT, Yoshimoto M, McGrath K, Conway SJ, Palis J & Yoder MC (2008) All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac. *Blood* **111**: 3435-3438.
Ma X, Lin WY, Chen Y, Stawicki S, Mukhyala K, Wu Y, Martin F, Bazan JF & Starovasnik MA (2012) Structural basis for the dual recognition of helical cytokines IL-34 and CSF-1 by CSF-1R. *Structure (London, England : 1993)* **20**: 676-687.
Mabbott NA, Kenneth Baillie J, Hume DA & Freeman TC (2010) Meta-analysis of lineage-specific gene expression signatures in mouse leukocyte populations. *Immunobiology* **215**: 724-736.

Mabbott NA, Baillie JK, Brown H, Freeman TC & Hume DA (2013) An expression atlas of human primary cells: inference of gene function from coexpression networks. *BMC genomics* **14**: 632.
Mabbott NA, Kenneth Baillie J, Kobayashi A, Donaldson DS, Ohmori H, Yoon SO, Freedman AS, Freeman TC & Summers KM (2011) Expression of mesenchyme-specific gene signatures by follicular dendritic cells: insights from the meta-analysis of microarray data from multiple mouse cell populations. *Immunology* **133**: 482-498.
MacDonald KP, Palmer JS, Cronau S*, et al.* (2010) An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood* **116**: 3955-3963.
MacHugh DE, Taraktsoglou M, Killick KE, Nalpas NC, Browne JA, DE Park S, Hokamp K, Gormley E & Magee DA (2012) Pan-genomic analysis of bovine monocyte-derived macrophage gene expression in response to in vitro infection with Mycobacterium avium subspecies paratuberculosis. *Veterinary research* **43**: 25.
Malandrino MI, Fucho R, Weber M*, et al.* (2015) Enhanced fatty acid oxidation in adipocytes and macrophages reduces lipid-induced triglyceride accumulation and inflammation. *American journal of physiology Endocrinology and metabolism* **308**: E756-769.
Malone KM, Rue-Albrecht K, Magee DA*, et al.* (2018) Comparative 'omics analyses differentiate Mycobacterium tuberculosis and Mycobacterium bovis and reveal distinct macrophage responses to infection with the human and bovine tubercle bacilli. *Microbial genomics*.
Man K, Loudon A & Chawla A (2016) Immunity around the clock. *Science (New York, NY)* **354**: 999-1003.
Mantovani A, Biswas SK, Galdiero MR, Sica A & Locati M (2013) Macrophage plasticity and polarization in tissue repair and remodelling. *The Journal of pathology* **229**: 176-185.
Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K & McKenna HJ (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *The Journal of experimental medicine* **184**: 1953-1962.
Marenholz I, Heizmann CW & Fritz G (2004) S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochemical and biophysical research communications* **322**: 1111-1122.
Margulies M, Egholm M, Altman WE*, et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376.
Marino R, Capoferri R, Panelli S, Minozzi G, Strozzi F, Trevisi E, Snel GGM, Ajmone-Marsan P & Williams JL (2017) Johne's disease in cattle: an in vitro model to study early response to infection of Mycobacterium avium subsp. paratuberculosis using RNA-seq. *Molecular immunology* **91**: 259-271.
Marr KJ, Jones GJ & Mody CH (2006) Contemplating the murine test tube: lessons from natural killer cells and Cryptococcus neoformans. *FEMS yeast research* **6**: 543-557.
Martineau HM, Cousens C, Imlach S, Dagleish MP & Griffiths DJ (2011) Jaagsiekte sheep retrovirus infects multiple cell types in the ovine lung. *Journal of virology* **85**: 3341-3355.
Martinez FO, Gordon S, Locati M & Mantovani A (2006) Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *Journal of immunology (Baltimore, Md : 1950)* **177**: 7303-7311.
Martinez FO, Helming L, Milde R*, et al.* (2013) Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood* **121**: e57-69.

Martinon F, Burns K & Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell* **10**: 417-426.
Martinon F, Chen X, Lee A-H & Glimcher LH (2010) TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nature immunology* **11**: 411-418.
Marzluff WF, Wagner EJ & Duronio RJ (2008) Metabolism and regulation of canonical histone mRNAs: life without a poly(A) tail. *Nature reviews Genetics* **9**: 843-854.
Mass E, Ballesteros I, Farlik M*, et al.* (2016) Specification of tissue-resident macrophages during organogenesis. *Science (New York, NY)* **353**.
Mattick JS (2001) Non-coding RNAs: the architects of eukaryotic complexity. *EMBO Rep* **2**: 986-991.
Maurano MT, Humbert R, Rynes E*, et al.* (2012) Systematic localization of common disease-associated variation in regulatory DNA. *Science (New York, NY)* **337**: 1190-1195.
Mazzone M, Menga A & Castegna A (2017) Metabolism and TAM functions-it takes two to tango. *The FEBS journal*.
McDermott JE, Vartanian KB, Mitchell H, Stevens SL, Sanfilippo A & Stenzel-Poore MP (2012) Identification and validation of Ifit1 as an important innate immune bottleneck. *PloS one* **7**: e36465.
McGinnis S & Madden TL (2004) BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic acids research* **32**: W20-25.
McKercher SR, Torbett BE, Anderson KL*, et al.* (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal* **15**: 5647-5658.
McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, Cowley AP & Lopez R (2013) Analysis Tool Web Services from the EMBL-EBI. *Nucleic acids research* **41**: W597-600.
Medzhitov R (2008) Origin and physiological roles of inflammation. *Nature* **454**: 428-435.
Medzhitov R (2010) Inflammation 2010: new adventures of an old flame. *Cell* **140**: 771-776.
Medzhitov R & Janeway CA, Jr. (1997) Innate Immunity: The Virtues of a Nonclonal System of Recognition. *Cell* **91**: 295-298.
Medzhitov R, Preston-Hurlburt P & Janeway CA, Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**: 394-397.
Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S & Janeway CA, Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Molecular cell* **2**: 253-258.
Meijer GA, van der Meulen J & van Vuuren AM (1993) Glutamine is a potentially limiting amino acid for milk production in dairy cows: a hypothesis. *Metabolism: clinical and experimental* **42**: 358-364.
Melamed P, Yosefzon Y, Rudnizky S & Pnueli L (2016) Transcriptional enhancers: Transcription, function and flexibility. *Transcription* **7**: 26-31.
Memczak S, Jens M, Elefsinioti A*, et al.* (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* **495**: 333-338.
Mestas J & Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. *Journal of immunology (Baltimore, Md : 1950)* **172**: 2731-2738.
Metcalf D (2008) Hematopoietic cytokines. *Blood* **111**: 485-491.
Metcalf D (2016) Growth and Differentiation Factors. *Microbiology spectrum* **4**.
Miar Y, Salehi A, Kolbehdari D & Aleyasin SA (2014) Application of myostatin in sheep breeding programs: A review. *Molecular biology research communications* **3**: 33-43.
Michelucci A, Cordes T, Ghelfi J*, et al.* (2013) Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 7820-7825.

Miller MB & Tang YW (2009) Basic concepts of microarrays and potential applications in clinical microbiology. *Clinical microbiology reviews* **22**: 611-633.
Miller SI, Ernst RK & Bader MW (2005) LPS, TLR4 and infectious disease diversity. *Nature Reviews Microbiology* **3**: 36.
Millet P, Vachharajani V, McPhail L, Yoza B & McCall CE (2016) GAPDH Binding to TNF-alpha mRNA Contributes to Posttranscriptional Repression in Monocytes: A Novel Mechanism of Communication between Inflammation and Metabolism. *Journal of immunology (Baltimore, Md : 1950)* **196**: 2541-2551.
Mills EL & O'Neill LA (2016) Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *European journal of immunology* **46**: 13-21.
Mills EL, Ryan DG, Prag HA*, et al.* (2018) Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature* **556**: 113-117.
Mink M, Fogelgren B, Olszewski K, Maroy P & Csiszar K (2001) A novel human gene (SARM) at chromosome 17q11 encodes a protein with a SAM motif and structural similarity to Armadillo/beta-catenin that is conserved in mouse, Drosophila, and Caenorhabditis elegans. *Genomics* **74**: 234-244.
Misharin AV, Morales-Nebreda L, Mutlu GM, Budinger GRS & Perlman H (2013) Flow Cytometric Analysis of Macrophages and Dendritic Cell Subsets in the Mouse Lung. *American Journal of Respiratory Cell and Molecular Biology* **49**: 503-510.
Montgomery GW & Sise JA (1990) Extraction of DNA from sheep white blood cells. *New Zealand Journal of Agricultural Research* **33**: 437-441.
Moon JS, Hisata S, Park MA, DeNicola GM, Ryter SW, Nakahira K & Choi AMK (2015) mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. *Cell reports* **12**: 102-115.
Moore KJ, Sheedy FJ & Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. *Nature reviews Immunology* **13**: 709-721.
Moore MJ & Proudfoot NJ (2009) Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell* **136**: 688-700.
Mortazavi A, Williams BA, McCue K, Schaeffer L & Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5**: 621-628.
Mosser DM & Zhang X (2008) Activation of murine macrophages. *Current protocols in immunology / edited by John E Coligan [et al]* **Chapter 14**: Unit 14.12.
Mosser DM & Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nature reviews Immunology* **8**: 958-969.
Mowat AM (2003) Anatomical basis of tolerance and immunity to intestinal antigens. *Nature reviews Immunology* **3**: 331-341.
Moyron-Quiroz JE, Partida-Sanchez S, Donis-Hernandez R, Sandoval-Montes C & Santos-Argumedo L (2002) Expression and function of CD22, a B-cell restricted molecule. *Scandinavian journal of immunology* **55**: 343-351.
Munder M, Mallo M, Eichmann K & Modolell M (1998) Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *The Journal of experimental medicine* **187**: 2103-2108.
Murray PJ & Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. *Nature reviews Immunology* **11**: 723-737.
Murray PJ, Rathmell J & Pearce E (2015) SnapShot: Immunometabolism. *Cell metabolism* **22**: 190-190.e191.
Murray PJ, Allen JE, Biswas SK*, et al.* (2014) Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41**: 14-20.

Murray SM, Picker LJ, Axthelm MK, Hudkins K, Alpers CE & Linial ML (2008) Replication in a superficial epithelial cell niche explains the lack of pathogenicity of primate foamy virus infections. *J Virol* **82**: 5981-5985.
Mutz KO, Heilkenbrinker A, Lonne M, Walter JG & Stahl F (2013) Transcriptome analysis using next-generation sequencing. *Current opinion in biotechnology* **24**: 22-30.
Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M & Miyake K (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature immunology* **3**: 667-672.
Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M & Snyder M (2008) The transcriptional landscape of the yeast genome defined by RNA sequencing. *Science (New York, NY)* **320**: 1344-1349.
Nagy E & Rigby WF (1995) Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rossmann fold). *The Journal of biological chemistry* **270**: 2755-2763.
Nakashima A, Carrero JJ, Qureshi AR, Miyamoto T, Anderstam B, Barany P, Heimburger O, Stenvinkel P & Lindholm B (2010) Effect of circulating soluble receptor for advanced glycation end products (sRAGE) and the proinflammatory RAGE ligand (EN-RAGE, S100A12) on mortality in hemodialysis patients. *Clinical journal of the American Society of Nephrology : CJASN* **5**: 2213-2219.
Nandi S, Gokhan S, Dai XM, Wei S, Enikolopov G, Lin H, Mehler MF & Stanley ER (2012) The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation. *Developmental biology* **367**: 100-113.
Nandi S, Cioce M, Yeung YG*, et al.* (2013) Receptor-type protein-tyrosine phosphatase zeta is a functional receptor for interleukin-34. *The Journal of biological chemistry* **288**: 21972-21986.
Nathan C (2002) Points of control in inflammation. *Nature* **420**: 846-852.
Natoli G (2010) Maintaining cell identity through global control of genomic organization. *Immunity* **33**: 12-24.
Nauseef WM & Borregaard N (2014) Neutrophils at work. *Nature immunology* **15**: 602-611.
Naville M, Warren IA, Haftek-Terreau Z, Chalopin D, Brunet F, Levin P, Galiana D & Volff JN (2016) Not so bad after all: retroviruses and long terminal repeat retrotransposons as a source of new genes in vertebrates. *Clinical Microbiology and Infection* **22**: 312-323.
Netea MG, Quintin J & van der Meer JW (2011) Trained immunity: a memory for innate host defense. *Cell host & microbe* **9**: 355-361.
Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, O'Neill LA & Xavier RJ (2016) Trained immunity: A program of innate immune memory in health and disease. *Science (New York, NY)* **352**: aaf1098.
Nicklin P, Bergman P, Zhang B*, et al.* (2009) Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* **136**: 521-534.
Nieto JE, MacDonald MH, Braim AE & Aleman M (2009) Effect of lipopolysaccharide infusion on gene expression of inflammatory cytokines in normal horses in vivo. *Equine veterinary journal* **41**: 717-719.
Nilsson R, Bajic VB, Suzuki H*, et al.* (2006) Transcriptional network dynamics in macrophage activation. *Genomics* **88**: 133-142.
Nimer SD & Uchida H (1995) Regulation of granulocyte-macrophage colony-stimulating factor and interleukin 3 expression. *Stem cells (Dayton, Ohio)* **13**: 324-335.
Nischalke HD, Berger C, Aldenhoff K*, et al.* (2011) Toll-like receptor (TLR) 2 promoter and intron 2 polymorphisms are associated with increased risk for spontaneous bacterial peritonitis in liver cirrhosis. *Journal of hepatology* **55**: 1010-1016.

Nitsche A, Junghahn I, Thulke S, Aumann J, Radonic A, Fichtner I & Siegert W (2003) Interleukin-3 promotes proliferation and differentiation of human hematopoietic stem cells but reduces their repopulation potential in NOD/SCID mice. *Stem cells (Dayton, Ohio)* **21**: 236-244.
Noguchi S & Arakawa T & Fukuda S*, et al.* (2017) FANTOM5 CAGE profiles of human and mouse samples. *Scientific data* **4**: 170112.
O'Neil D, Glowatz H & Schlumpberger M (2013) Ribosomal RNA depletion for efficient use of RNA-seq capacity. *Current protocols in molecular biology* **Chapter 4**: Unit 4.19.
O'Neill LA & Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature reviews Immunology* **7**: 353-364.
O'Neill LA, Kishton RJ & Rathmell J (2016) A guide to immunometabolism for immunologists. *Nature reviews Immunology* **16**: 553-565.
Ogi K, Miyamoto Y, Masuda Y, Habata Y, Hosoya M, Ohtaki T, Masuo Y, Onda H & Fujino M (1993) Molecular cloning and functional expression of a cDNA encoding a human pituitary adenylate cyclase activating polypeptide receptor. *Biochemical and biophysical research communications* **196**: 1511-1521.
Oliver S (2000) Guilt-by-association goes global. *Nature* **403**: 601-603.
Onami J, Nikaido M, Mannen H & Okada N (2007) Genomic expansion of the Bov-A2 retroposon relating to phylogeny and breed management. *Mammalian genome : official journal of the International Mammalian Genome Society* **18**: 187-196.
Orkin SH & Zon LI (2008) Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell* **132**: 631-644.
Oshlack A, Robinson MD & Young MD (2010) From RNA-seq reads to differential expression results. *Genome biology* **11**: 220.
Ostuni R & Natoli G (2011) Transcriptional control of macrophage diversity and specialization. *European journal of immunology* **41**: 2486-2490.
Otero DC, Anzelon AN & Rickert RC (2003) CD19 function in early and late B cell development: I. Maintenance of follicular and marginal zone B cells requires CD19-dependent survival signals. *Journal of immunology (Baltimore, Md : 1950)* **170**: 73-83.
Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH & Hotamisligil GS (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science (New York, NY)* **306**: 457-461.
Ozsolak F & Milos PM (2011) RNA sequencing: advances, challenges and opportunities. *Nature reviews Genetics* **12**: 87-98.
Palmer C, Diehn M, Alizadeh AA & Brown PO (2006) Cell-type specific gene expression profiles of leukocytes in human peripheral blood. *BMC genomics* **7**: 115-115.
Palmieri EM, Menga A, Martin-Perez R*, et al.* (2017) Pharmacologic or Genetic Targeting of Glutamine Synthetase Skews Macrophages toward an M1-like Phenotype and Inhibits Tumor Metastasis. *Cell reports* **20**: 1654-1666.
Palsson-McDermott EM, Curtis AM, Goel G*, et al.* (2015) Pyruvate Kinase M2 Regulates Hif-1alpha Activity and IL-1beta Induction and Is a Critical Determinant of the Warburg Effect in LPS-Activated Macrophages. *Cell metabolism* **21**: 347.
Palu RAS, Praggastis SA & Thummel CS (2017) Parental obesity leads to metabolic changes in the F2 generation in Drosophila. *Molecular Metabolism* **6**: 631-639.
Palucka K & Banchereau J (1999) Linking innate and adaptive immunity. *Nature medicine* **5**: 868-870.
Pan Q, Shai O, Lee LJ, Frey BJ & Blencowe BJ (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature genetics* **40**: 1413-1415.

Papathanassiu AE, Ko J-H, Imprialou M*, et al.* (2017) BCAT1 controls metabolic reprogramming in activated human macrophages and is associated with inflammatory diseases. *Nature communications* **8**: 16040.
Park JM, Greten FR, Wong A, Westrick RJ, Arthur JS, Otsu K, Hoffmann A, Montminy M & Karin M (2005) Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis--CREB and NF-kappaB as key regulators. *Immunity* **23**: 319-329.
Pasare C & Medzhitov R (2005) Toll-like receptors: linking innate and adaptive immunity. *Advances in experimental medicine and biology* **560**: 11-18.
Patel JR & García-Sastre A (2014) Activation and regulation of pathogen sensor RIG-I. *Cytokine & Growth Factor Reviews* **25**: 513-523.
Paterson S, Vogwill T, Buckling A*, et al.* (2010) Antagonistic coevolution accelerates molecular evolution. *Nature* **464**: 275-278.
Paul William E (2011) Bridging Innate and Adaptive Immunity. *Cell* **147**: 1212-1215.
Perdiguero EG & Geissmann F (2016) The development and maintenance of resident macrophages. *Nature immunology* **17**: 2-8.
Perlman RL (2016) Mouse models of human disease: An evolutionary perspective. *Evolution, Medicine, and Public Health* **2016**: 170-176.
Perocchi F, Xu Z, Clauder-Munster S & Steinmetz LM (2007) Antisense artifacts in transcriptome microarray experiments are resolved by actinomycin D. *Nucleic acids research* **35**: e128.
Pertea M, Kim D, Pertea GM, Leek JT & Salzberg SL (2016) Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nature protocols* **11**: 1650-1667.
Pertea M, Kim D, Pertea GM, Leek JT & Salzberg SL (2016) Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nature Protocols* **11**: 1650.
Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT & Salzberg SL (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature biotechnology* **33**: 290-295.
Pessanha AP, Martins RA, Mattos-Guaraldi AL, Vianna A & Moreira LO (2012) Arginase-1 expression in granulomas of tuberculosis patients. *FEMS immunology and medical microbiology* **66**: 265-268.
Picard C, Puel A, Bonnet M*, et al.* (2003) Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science (New York, NY)* **299**: 2076-2079.
Pierce KL, Premont RT & Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nature Reviews Molecular Cell Biology* **3**: 639.
Pimentel H, Bray NL, Puente S, Melsted P & Pachter L (2017) Differential analysis of RNA-seq incorporating quantification uncertainty. *Nat Methods* **14**: 687-690.
Piñero P, Juanola O, Caparrós E, Zapater P, Giménez P, González-Navajas JM, Such J & Francés R (2017) Toll-like receptor polymorphisms compromise the inflammatory response against bacterial antigen translocation in cirrhosis. *Scientific reports* **7**: 46425.
Pixley FJ & Stanley ER (2004) CSF-1 regulation of the wandering macrophage: complexity in action. *Trends in cell biology* **14**: 628-638.
Plain KM, de Silva K, Earl J, Begg DJ, Purdie AC & Whittington RJ (2011) Indoleamine 2,3-dioxygenase, tryptophan catabolism, and Mycobacterium avium subsp. paratuberculosis: a model for chronic mycobacterial infections. *Infection and immunity* **79**: 3821-3832.
Platanias LC (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nature reviews Immunology* **5**: 375-386.
Poltorak A, He X, Smirnova I*, et al.* (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science (New York, NY)* **282**: 2085-2088.

PolyA_mRNA_ILLUMINA (2013) TruSeq Stranded mRNA Sample Preparation Guide Part:15031047 Rev. E.
Qualls JE, Neale G, Smith AM, Koo MS, DeFreitas AA, Zhang H, Kaplan G, Watowich SS & Murray PJ (2010) Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. *Science signaling* **3**: ra62.
Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P & Malo D (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *The Journal of experimental medicine* **189**: 615-625.
R. McKercher S, Henkel G & Maki R (1999) *The transcription factor PU.1 does not regulate lineage commitment but has lineage-specific effects*.
Ragland SA & Criss AK (2017) From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS pathogens* **13**: e1006512.
Rai SK, Duh F-M, Vigdorovich V, Danilkovitch-Miagkova A, Lerman MI & Miller AD (2001) Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 4443-4448.
Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S & Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* **118**: 229-241.
Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, Black JC, Hoffmann A, Carey M & Smale ST (2009) A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* **138**: 114-128.
Ramsey SA, Klemm SL, Zak DE*, et al.* (2008) Uncovering a macrophage transcriptional program by integrating evidence from motif scanning and expression dynamics. *PLoS computational biology* **4**: e1000021.
Rand DM (1994) Thermal habit, metabolic rate and the evolution of mitochondrial DNA. *Trends in Ecology & Evolution* **9**: 125-131.
Ravasi T, Wells CA & Hume DA (2007) Systems biology of transcription control in macrophages. *BioEssays : news and reviews in molecular, cellular and developmental biology* **29**: 1215-1226.
Ravasi T, Wells C, Forest A, Underhill DM, Wainwright BJ, Aderem A, Grimmond S & Hume DA (2002) Generation of diversity in the innate immune system: macrophage heterogeneity arises from gene-autonomous transcriptional probability of individual inducible genes. *Journal of immunology (Baltimore, Md : 1950)* **168**: 44-50.
Raza S, Barnett MW, Barnett-Itzhaki Z, Amit I, Hume DA & Freeman TC (2014) Analysis of the transcriptional networks underpinning the activation of murine macrophages by inflammatory mediators. *Journal of leukocyte biology* **96**: 167-183.
Regan T, Gill AC, Clohisey SM, Barnett MW, Pariante CM, Harrison NA, Hume DA, Bullmore ET & Freeman TC (2018) Effects of anti-inflammatory drugs on the expression of tryptophan-metabolism genes by human macrophages. *Journal of leukocyte biology*.
Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F & Glimcher LH (2001) Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412**: 300-307.
Ren K & Torres R (2009) Role of interleukin-1beta during pain and inflammation. *Brain research reviews* **60**: 57-64.
Renaud HJ, Cui JY, Lu H & Klaassen CD (2014) Effect of Diet on Expression of Genes Involved in Lipid Metabolism, Oxidative Stress, and Inflammation in Mouse Liver–Insights into Mechanisms of Hepatic Steatosis. *PloS one* **9**: e88584.

Reynolds CK & Huntington GB (1988) Partition of portal-drained visceral net flux in beef steers. 1. Blood flow and net flux of oxygen, glucose and nitrogenous compounds across stomach and post-stomach tissues. *The British journal of nutrition* **60**: 539-551.
Ribodepleted_totalRNA_ILLUMINA (2013) TruSeq Stranded Total RNA Sample Preparation Guide Part: 15031048, Rev. E. p.^pp.
Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE & Aderem A (2005) The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 9577-9582.
Roberts A, Pimentel H, Trapnell C & Pachter L (2011) Identification of novel transcripts in annotated genomes using RNA-Seq. *Bioinformatics (Oxford, England)* **27**: 2325-2329.
Roberts A, Trapnell C, Donaghey J, Rinn JL & Pachter L (2011) Improving RNA-Seq expression estimates by correcting for fragment bias. *Genome biology* **12**: R22.
Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G & Mesirov JP (2011) Integrative genomics viewer. *Nature biotechnology* **29**: 24-26.
Rojo R, Pridans C, Langlais D & Hume DA (2017) Transcriptional mechanisms that control expression of the macrophage colony-stimulating factor receptor locus. *Clinical science (London, England : 1979)* **131**: 2161-2182.
Rooks MG & Garrett WS (2016) Gut microbiota, metabolites and host immunity. *Nature reviews Immunology* **16**: 341-352.
Rosselot AE, Hong CI & Moore SR (2016) Rhythm and bugs: circadian clocks, gut microbiota, and enteric infections. *Current opinion in gastroenterology* **32**: 7-11.
Rual JF, Venkatesan K, Hao T*, et al.* (2005) Towards a proteome-scale map of the human protein-protein interaction network. *Nature* **437**: 1173-1178.
Rubic T, Lametschwandtner G, Jost S*, et al.* (2008) Triggering the succinate receptor GPR91 on dendritic cells enhances immunity. *Nature immunology* **9**: 1261-1269.
Ruckebusch Y & Thivend P (2012) *Digestive Physiology and Metabolism in Ruminants: Proceedings of the 5th International Symposium on Ruminant Physiology, held at Clermont — Ferrand, on 3rd–7th September, 1979*. Springer Netherlands.
Ruprecht RM, Goodman NC & Spiegelman S (1973) Conditions for the selective synthesis of DNA complementary to template RNA. *Biochimica et biophysica acta* **294**: 192-203.
Russell JB & Rychlik JL (2001) Factors that alter rumen microbial ecology. *Science (New York, NY)* **292**: 1119-1122.
Russo-Marie F (1992) Macrophages and the glucocorticoids. *Journal of Neuroimmunology* **40**: 281-286.
Ryan DG & O'Neill LAJ (2017) Krebs cycle rewired for macrophage and dendritic cell effector functions. *FEBS letters* **591**: 2992-3006.
Saccani S, Pantano S & Natoli G (2001) Two waves of nuclear factor kappaB recruitment to target promoters. *The Journal of experimental medicine* **193**: 1351-1359.
Sacerdote P, Massi P, Panerai AE & Parolaro D (2000) In vivo and in vitro treatment with the synthetic cannabinoid CP55, 940 decreases the in vitro migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. *J Neuroimmunol* **109**: 155-163.
Sale MM, Smith SG, Mychaleckyj JC, Keene KL, Langefeld CD, Leak TS, Hicks PJ, Bowden DW, Rich SS & Freedman BI (2007) Variants of the transcription factor 7-like 2 (TCF7L2) gene are associated with type 2 diabetes in an African-American population enriched for nephropathy. *Diabetes* **56**: 2638-2642.
Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH & Jacobson MA (2000) Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *The Journal of biological chemistry* **275**: 4429-4434.
Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, Ostrowski MC, Himes SR & Hume DA (2003) A macrophage colony-stimulating factor receptor-green fluorescent

protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* **101**: 1155-1163.
Sauter KA, Bouhlel MA, O'Neal J, Sester DP, Tagoh H, Ingram RM, Pridans C, Bonifer C & Hume DA (2013) The function of the conserved regulatory element within the second intron of the mammalian Csf1r locus. *PloS one* **8**: e54935.
Sauter KA, Pridans C, Sehgal A*, et al.* (2014) Pleiotropic effects of extended blockade of CSF1R signaling in adult mice. *Journal of leukocyte biology* **96**: 265-274.
Schieber M & Chandel NS (2014) ROS function in redox signaling and oxidative stress. *Current biology : CB* **24**: R453-R462.
Schmidl C, Renner K, Peter K*, et al.* (2014) Transcription and enhancer profiling in human monocyte subsets. *Blood* **123**: e90-99.
Schnare M, Barton GM, Holt AC, Takeda K, Akira S & Medzhitov R (2001) Toll-like receptors control activation of adaptive immune responses. *Nature immunology* **2**: 947-950.
Schneemann M & Schoeden G (2007) Macrophage biology and immunology: man is not a mouse. *Journal of leukocyte biology* **81**: 579; discussion 580.
Schneider C, Nobs SP, Kurrer M, Rehrauer H, Thiele C & Kopf M (2014) Induction of the nuclear receptor PPAR-γ by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages. *Nature immunology* **15**: 1026.
Schroder K & Tschopp J (2010) The inflammasomes. *Cell* **140**: 821-832.
Schroder K, Hertzog PJ, Ravasi T & Hume DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of leukocyte biology* **75**: 163-189.
Schroder K, Irvine KM, Taylor MS*, et al.* (2012) Conservation and divergence in Toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **109**: E944-953.
Schroder WA, Gardner J, Le TT, Duke M, Burke ML, Jones MK, McManus DP & Suhrbier A (2010) SerpinB2 deficiency modulates Th1Th2 responses after schistosome infection. *Parasite immunology* **32**: 764-768.
Schromm AB, Brandenburg K, Loppnow H, Moran AP, Koch MH, Rietschel ET & Seydel U (2000) Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion. *European journal of biochemistry* **267**: 2008-2013.
Schromm AB, Brandenburg K, Loppnow H, Zahringer U, Rietschel ET, Carroll SF, Koch MH, Kusumoto S & Seydel U (1998) The charge of endotoxin molecules influences their conformation and IL-6-inducing capacity. *Journal of immunology (Baltimore, Md : 1950)* **161**: 5464-5471.
Schulz C, Gomez Perdiguero E, Chorro L*, et al.* (2012) A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science (New York, NY)* **336**: 86-90.
Scott EW, Simon MC, Anastasi J & Singh H (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science (New York, NY)* **265**: 1573-1577.
Scott WA, Pawlowski NA, Murray HW, Andreach M, Zrike J & Cohn ZA (1982) Regulation of arachidonic acid metabolism by macrophage activation. *The Journal of experimental medicine* **155**: 1148-1160.
Seabra SH, DaMatta RA, de Mello FG & de Souza W (2004) Endogenous polyamine levels in macrophages is sufficient to support growth of Toxoplasma gondii. *The Journal of parasitology* **90**: 455-460.
Semenza GL, Roth PH, Fang HM & Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *The Journal of biological chemistry* **269**: 23757-23763.

Seok J, Warren HS, Cuenca AG*, et al.* (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 3507-3512.
Sester DP, Beasley SJ, Sweet MJ, Fowles LF, Cronau SL, Stacey KJ & Hume DA (1999) Bacterial/CpG DNA down-modulates colony stimulating factor-1 receptor surface expression on murine bone marrow-derived macrophages with concomitant growth arrest and factor-independent survival. *Journal of immunology (Baltimore, Md : 1950)* **163**: 6541-6550.
Sester DP, Trieu A, Brion K*, et al.* (2005) LPS regulates a set of genes in primary murine macrophages by antagonising CSF-1 action. *Immunobiology* **210**: 97-107.
Shabat SKB, Sasson G, Doron-Faigenboim A, Durman T, Yaacoby S, Berg Miller ME, White BA, Shterzer N & Mizrahi I (2016) Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The Isme Journal* **10**: 2958.
Shaw MH, Reimer T, Kim YG & Nunez G (2008) NOD-like receptors (NLRs): bona fide intracellular microbial sensors. *Current opinion in immunology* **20**: 377-382.
Shea-Donohue T, Zhao A & Antalis TM (2014) SerpinB2 mediated regulation of macrophage function during enteric infection. *Gut microbes* **5**: 254-258.
Shibata Y, Berclaz P-Y, Chroneos ZC, Yoshida M, Whitsett JA & Trapnell BC (2001) GM-CSF Regulates Alveolar Macrophage Differentiation and Innate Immunity in the Lung through PU.1. *Immunity* **15**: 557-567.
Sierra-Filardi E, Nieto C, Dominguez-Soto A*, et al.* (2014) CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *Journal of immunology (Baltimore, Md : 1950)* **192**: 3858-3867.
Sievers F, Wilm A, Dineen D*, et al.* (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* **7**: 539.
Singh K, Al-Greene NT, Verriere TG*, et al.* (2016) The L-Arginine Transporter Solute Carrier Family 7 Member 2 Mediates the Immunopathogenesis of Attaching and Effacing Bacteria. *PLoS pathogens* **12**: e1005984.
Smedley D & Haider S & Durinck S*, et al.* (2015) The BioMart community portal: an innovative alternative to large, centralized data repositories. *Nucleic acids research* **43**: W589-598.
Smirnova I, Poltorak A, Chan EKL, McBride C & Beutler B (2000) Phylogenetic variation and polymorphism at the Toll-like receptor 4 locus (TLR4). *Genome biology* **1**: research002.001-research002.010.
Smirnova I, Hamblin MT, McBride C, Beutler B & Di Rienzo A (2001) Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans. *Genetics* **158**: 1657-1664.
Smirnova I, Mann N, Dols A, Derkx HH, Hibberd ML, Levin M & Beutler B (2003) Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 6075-6080.
Soares MP & Hamza I (2016) Macrophages and Iron Metabolism. *Immunity* **44**: 492-504.
Sordet O, Rebe C, Plenchette S, Zermati Y, Hermine O, Vainchenker W, Garrido C, Solary E & Dubrez-Daloz L (2002) Specific involvement of caspases in the differentiation of monocytes into macrophages. *Blood* **100**: 4446-4453.
Stanley ER & Heard PM (1977) Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *The Journal of biological chemistry* **252**: 4305-4312.
Stanley ER & Chitu V (2014) CSF-1 receptor signaling in myeloid cells. *Cold Spring Harbor perspectives in biology* **6**.

Stelzer G, Rosen N, Plaschkes I*, et al.* (2016) The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. *Current protocols in bioinformatics* **54**: 1.30.31-31.30.33.
Stienstra R, Netea-Maier RT, Riksen NP, Joosten LAB & Netea MG (2017) Specific and Complex Reprogramming of Cellular Metabolism in Myeloid Cells during Innate Immune Responses. *Cell metabolism* **26**: 142-156.
Stoye JP (2012) Studies of endogenous retroviruses reveal a continuing evolutionary saga. *Nature reviews Microbiology* **10**: 395-406.
Sultan M, Amstislavskiy V, Risch T, Schuette M, Dökel S, Ralser M, Balzereit D, Lehrach H & Yaspo M-L (2014) Influence of RNA extraction methods and library selection schemes on RNA-seq data. *BMC genomics* **15**: 675.
Sun L, Zhang Z, Bailey TL, Perkins AC, Tallack MR, Xu Z & Liu H (2012) Prediction of novel long non-coding RNAs based on RNA-Seq data of mouse Klf1 knockout study. *BMC Bioinformatics* **13**: 331-331.
Sundling C & Sandgren K (2016) CTI special feature on innate immune responses and vaccine design. *Clin Trans Immunol* **5**: e96.
Sweet MJ & Hume DA (1996) Endotoxin signal transduction in macrophages. *Journal of leukocyte biology* **60**: 8-26.
Sweet MJ, Campbell CC, Sester DP, Xu D, McDonald RC, Stacey KJ, Hume DA & Liew FY (2002) Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. *Journal of immunology (Baltimore, Md : 1950)* **168**: 392-399.
Swirski FK, Hilgendorf I & Robbins CS (2014) From proliferation to proliferation: monocyte lineage comes full circle. *Seminars in immunopathology* **36**: 137-148.
Tabeta K, Hoebe K, Janssen EM*, et al.* (2006) The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nature immunology* **7**: 156-164.
Tagliani E, Shi C, Nancy P, Tay C-S, Pamer EG & Erlebacher A (2011) Coordinate regulation of tissue macrophage and dendritic cell population dynamics by CSF-1. *The Journal of experimental medicine* **208**: 1901.
Takao K & Miyakawa T (2015) Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 1167-1172.
Takeda K & Akira S (2015) Toll-like receptors. *Current protocols in immunology / edited by John E Coligan [et al]* **109**: 14.12.11-14.12.10.
Takeuchi O & Akira S (2010) Pattern recognition receptors and inflammation. *Cell* **140**: 805-820.
Tan Z, Xie N, Banerjee S, Cui H, Fu M, Thannickal VJ & Liu G (2015) The monocarboxylate transporter 4 is required for glycolytic reprogramming and inflammatory response in macrophages. *The Journal of biological chemistry* **290**: 46-55.
Tang T, Scambler TE, Smallie T, Cunliffe HE, Ross EA, Rosner DR, O'Neil JD & Clark AR (2017) Macrophage responses to lipopolysaccharide are modulated by a feedback loop involving prostaglandin E(2), dual specificity phosphatase 1 and tristetraprolin. *Scientific reports* **7**: 4350.
Tannahill GM, Curtis AM, Adamik J*, et al.* (2013) Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* **496**: 238-242.
Tarling JD, Lin HS & Hsu S (1987) Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies. *Journal of leukocyte biology* **42**: 443-446.
Tauber AI (2003) Metchnikoff and the phagocytosis theory. *Nature Reviews Molecular Cell Biology* **4**: 897.

Tavakoli S, Short JD, Downs K, Nguyen HN, Lai Y, Zhang W, Jerabek P, Goins B, Sadeghi MM & Asmis R (2017) Differential Regulation of Macrophage Glucose Metabolism by Macrophage Colony-stimulating Factor and Granulocyte-Macrophage Colony-stimulating Factor: Implications for (18)F FDG PET Imaging of Vessel Wall Inflammation. *Radiology* **283**: 87-97.
Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD & Gordon S (2005) Macrophage receptors and immune recognition. *Annual review of immunology* **23**: 901-944.
Taylor PR, Tsoni SV, Willment JA*, et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nature immunology* **8**: 31-38.
Temin HM (1989) Reverse transcriptases. Retrons in bacteria. *Nature* **339**: 254-255.
Temin HM (1992) Origin and General Nature of Retroviruses. *The Retroviridae,*(Levy JA, ed.) p.^pp. 1-18. Springer US, Boston, MA.
Temin HM & Mizutani S (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* **226**: 1211-1213.
Theocharidis A, van Dongen S, Enright AJ & Freeman TC (2009) Network visualization and analysis of gene expression data using BioLayout Express(3D). *Nature protocols* **4**: 1535-1550.
Theurl I, Hilgendorf I, Nairz M*, et al.* (2016) On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nature medicine* **22**: 945-951.
Thomas SJ, Snowden JA, Zeidler MP & Danson SJ (2015) The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. *British Journal Of Cancer* **113**: 365.
Thompson RW, Pesce JT, Ramalingam T*, et al.* (2008) Cationic amino acid transporter-2 regulates immunity by modulating arginase activity. *PLoS pathogens* **4**: e1000023.
Thorvaldsdottir H, Robinson JT & Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in bioinformatics* **14**: 178-192.
Torok HP, Glas J, Tonenchi L, Mussack T & Folwaczny C (2004) Polymorphisms of the lipopolysaccharide-signaling complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis. *Clinical immunology (Orlando, Fla)* **112**: 85-91.
Trapnell C, Pachter L & Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics (Oxford, England)* **25**: 1105-1111.
Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ & Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology* **28**: 511-515.
Trynka G, Sandor C, Han B, Xu H, Stranger BE, Liu XS & Raychaudhuri S (2013) Chromatin marks identify critical cell types for fine mapping complex trait variants. *Nature genetics* **45**: 124-130.
Ueda HR, Hayashi S, Matsuyama S, Yomo T, Hashimoto S, Kay SA, Hogenesch JB & Iino M (2004) Universality and flexibility in gene expression from bacteria to human. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 3765-3769.
Ueki K & Kadowaki T (2011) The other sweet face of XBP-1. *Nature medicine* **17**: 246-248.
Upton KR, Baillie JK & Faulkner GJ (2011) Is somatic retrotransposition a parasitic or symbiotic phenomenon? *Mobile genetic elements* **1**: 279-282.
Valles I, Pajares MJ, Segura V, Guruceaga E, Gomez-Roman J, Blanco D, Tamura A, Montuenga LM & Pio R (2012) Identification of novel deregulated RNA metabolism-related genes in non-small cell lung cancer. *PloS one* **7**: e42086.

Van den Bossche J, Baardman J, Otto NA*, et al.* (2016) Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. *Cell reports* **17**: 684-696.
van der Heijden C, Noz MP, Joosten LAB, Netea MG, Riksen NP & Keating ST (2018) Epigenetics and Trained Immunity. *Antioxidants & redox signaling* **29**: 1023-1040.
Van Dyken SJ & Locksley RM (2013) Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. *Annual review of immunology* **31**: 317-343.
Vandal K, Rouleau P, Boivin A, Ryckman C, Talbot M & Tessier PA (2003) Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. *Journal of immunology (Baltimore, Md : 1950)* **171**: 2602-2609.
Varro A, Noble PJ, Pritchard DM, Kennedy S, Hart CA, Dimaline R & Dockray GJ (2004) Helicobacter pylori induces plasminogen activator inhibitor 2 in gastric epithelial cells through nuclear factor-kappaB and RhoA: implications for invasion and apoptosis. *Cancer research* **64**: 1695-1702.
Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A & Vaudry H (2000) Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacological reviews* **52**: 269-324.
Vaure C & Liu Y (2014) A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species. *Frontiers in immunology* **5**: 316.
Vautier S, MacCallum DM & Brown GD (2012) C-type lectin receptors and cytokines in fungal immunity. *Cytokine* **58**: 89-99.
Velculescu VE, Zhang L, Vogelstein B & Kinzler KW (1995) Serial analysis of gene expression. *Science (New York, NY)* **270**: 484-487.
Vidal S, Tremblay ML, Govoni G, Gauthier S, Sebastiani G, Malo D, Skamene E, Olivier M, Jothy S & Gros P (1995) The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *The Journal of experimental medicine* **182**: 655-666.
Vidal SM, Malo D, Vogan K, Skamene E & Gros P (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* **73**: 469-485.
Vilella AJ, Severin J, Ureta-Vidal A, Heng L, Durbin R & Birney E (2009) EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome Res* **19**: 327-335.
Viljakainen L (2015) Evolutionary genetics of insect innate immunity. *Briefings in Functional Genomics* **14**: 407-412.
Visvader J & Verma IM (1989) Differential transcription of exon 1 of the human c-fms gene in placental trophoblasts and monocytes. *Molecular and cellular biology* **9**: 1336-1341.
Vogel Hertzel A & Bernlohr DA (2000) The Mammalian Fatty Acid-binding Protein Multigene Family: Molecular and Genetic Insights into Function. *Trends in Endocrinology & Metabolism* **11**: 175-180.
Vogl T, Tenbrock K, Ludwig S*, et al.* (2007) Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature medicine* **13**: 1042-1049.
Vogt PK (1997) Historical Introduction to the General Properties of Retroviruses. *Retroviruses,*(Coffin JM, Hughes SH & Varmus HE, eds.), p.^pp. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).
Waddell LA, Lefevre L, Bush SJ*, et al.* (2018) ADGRE1 (EMR1, F4/80) Is a Rapidly-Evolving Gene Expressed in Mammalian Monocyte-Macrophages. *Frontiers in immunology* **9**.
Wallace C & Keast D (1992) Glutamine and macrophage function. *Metabolism: clinical and experimental* **41**: 1016-1020.

Walsh AM, Kortschak RD, Gardner MG, Bertozzi T & Adelson DL (2013) Widespread horizontal transfer of retrotransposons. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 1012-1016.
Wanasen N, MacLeod CL, Ellies LG & Soong L (2007) L-arginine and cationic amino acid transporter 2B regulate growth and survival of Leishmania amazonensis amastigotes in macrophages. *Infection and immunity* **75**: 2802-2810.
Wang C, Yu X, Cao Q, Wang Y, Zheng G, Tan TK, Zhao H, Zhao Y, Wang Y & Harris DCH (2013) Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC immunology* **14**: 6-6.
Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C, Kingsmore SF, Schroth GP & Burge CB (2008) Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**: 470-476.
Wang L, Hu S, Liu Q, Li Y, Xu L, Zhang Z, Cai X & He X (2017) Porcine alveolar macrophage polarization is involved in inhibition of porcine reproductive and respiratory syndrome virus (PRRSV) replication. *The Journal of Veterinary Medical Science* **79**: 1906-1915.
Wang X, Liu J, Zhou G*, et al.* (2016) Whole-genome sequencing of eight goat populations for the detection of selection signatures underlying production and adaptive traits. *Scientific reports* **6**: 38932.
Wang Z, Gerstein M & Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews Genetics* **10**: 57-63.
Watanabe S & Uchida T (1995) Cloning and expression of human uridine phosphorylase. *Biochemical and biophysical research communications* **216**: 265-272.
Wei C, Jung J & Sanz I (2011) OMIP-003: phenotypic analysis of human memory B cells. *Cytometry Part A : the journal of the International Society for Analytical Cytology* **79**: 894-896.
Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, Williams LT, Lin H & Stanley ER (2010) Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *Journal of leukocyte biology* **88**: 495-505.
Wells CA, Ravasi T & Hume DA (2005) Inflammation suppressor genes: please switch out all the lights. *Journal of leukocyte biology* **78**: 9-13.
Wells CA, Ravasi T, Faulkner GJ, Carninci P, Okazaki Y, Hayashizaki Y, Sweet M, Wainwright BJ & Hume DA (2003) Genetic control of the innate immune response. *BMC immunology* **4**: 5.
Wells CA, Ravasi T, Sultana R*, et al.* (2003) Continued discovery of transcriptional units expressed in cells of the mouse mononuclear phagocyte lineage. *Genome research* **13**: 1360-1365.
Wenzel J, Held C, Palmisano R, Teufel S, David JP, Wittenberg T & Lang R (2011) Measurement of TLR-Induced Macrophage Spreading by Automated Image Analysis: Differential Role of Myd88 and MAPK in Early and Late Responses. *Frontiers in physiology* **2**: 71.
Wheelock EF (1965) Interferon-Like Virus-Inhibitor Induced in Human Leukocytes by Phytohemagglutinin. *Science (New York, NY)* **149**: 310-311.
Wicks IP & Roberts AW (2016) Targeting GM-CSF in inflammatory diseases. *Nature reviews Rheumatology* **12**: 37-48.
Wilkinson S, Bishop S, Allen AR, Skuce R, McBride SH, Bermingham M, Woolliams J & Glass E (2017) Fine-mapping host genetic variation underlying outcomes to Mycobacterium bovis infection in dairy cows. *BMC genomics* **18**.
Wilusz JE, Freier SM & Spector DL (2008) 3' end processing of a long nuclear-retained noncoding RNA yields a tRNA-like cytoplasmic RNA. *Cell* **135**: 919-932.

Wolf AJ, Reyes CN, Liang W*, et al.* (2016) Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan. *Cell* **166**: 624-636.
Wu Z, Liu X, Liu L, Deng H, Zhang J, Xu Q, Cen B & Ji A (2014) Regulation of lncRNA expression. *Cellular & Molecular Biology Letters* **19**: 561-575.
Wynn TA, Chawla A & Pollard JW (2013) Macrophage biology in development, homeostasis and disease. *Nature* **496**: 445-455.
Xia C, Braunstein Z, Toomey AC, Zhong J & Rao X (2017) S100 Proteins As an Important Regulator of Macrophage Inflammation. *Frontiers in immunology* **8**: 1908.
Xu Y, Tao X, Shen B, Horng T, Medzhitov R, Manley JL & Tong L (2000) Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature* **408**: 111-115.
Xue J, Schmidt SV, Sander J*, et al.* (2014) Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* **40**: 274-288.
Yamamoto M, Takeda K & Akira S (2004) TIR domain-containing adaptors define the specificity of TLR signaling. *Molecular immunology* **40**: 861-868.
Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K & Akira S (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *Journal of immunology (Baltimore, Md : 1950)* **169**: 6668-6672.
Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K & Akira S (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nature immunology* **4**: 1144-1150.
Yamamoto M, Sato S, Hemmi H*, et al.* (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science (New York, NY)* **301**: 640-643.
Yamamoto M, Sato S, Hemmi H*, et al.* (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* **420**: 324-329.
Yan SF, Ramasamy R & Schmidt AM (2010) The RAGE axis: a fundamental mechanism signaling danger to the vulnerable vasculature. *Circulation research* **106**: 842-853.
Yang L, Duff MO, Graveley BR, Carmichael GG & Chen L-L (2011) Genomewide characterization of non-polyadenylated RNAs. *Genome biology* **12**: R16.
Yang WS & Stockwell BR (2016) Ferroptosis: death by lipid peroxidation. *Trends in cell biology* **26**: 165-176.
Yao D & Brownlee M (2010) Hyperglycemia-Induced Reactive Oxygen Species Increase Expression of the Receptor for Advanced Glycation End Products (RAGE) and RAGE Ligands. *Diabetes* **59**: 249-255.
Yayar O, Eser B, Buyukbakkal M*, et al.* (2015) Do elevated plasma S100A12 levels predict atherosclerosis in peritoneal dialysis patients? *Renal failure* **37**: 845-850.
Ye D, Li FY, Lam KS, Li H, Jia W, Wang Y, Man K, Lo CM, Li X & Xu A (2012) Toll-like receptor-4 mediates obesity-induced non-alcoholic steatohepatitis through activation of X-box binding protein-1 in mice. *Gut* **61**: 1058-1067.
Ye J, McGinnis S & Madden TL (2006) BLAST: improvements for better sequence analysis. *Nucleic acids research* **34**: W6-9.
Yeramian A, Martin L, Serrat N*, et al.* (2006) Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. *Journal of immunology (Baltimore, Md : 1950)* **176**: 5918-5924.
Yin Q-F, Yang L, Zhang Y, Xiang J-F, Wu Y-W, Carmichael Gordon G & Chen L-L (2012) Long Noncoding RNAs with snoRNA Ends. *Molecular cell* **48**: 219-230.
Yon L, Breault L, Contesse V, Bellancourt G, Delarue C, Fournier A, Lehoux JG, Vaudry H & Gallo-Payet N (1998) Localization, characterization, and second messenger coupling of pituitary adenylate cyclase-activating polypeptide receptors in the fetal human adrenal

gland during the second trimester of gestation. *The Journal of clinical endocrinology and metabolism* **83**: 1299-1305.
Yona S, Kim KW, Wolf Y*, et al.* (2013) Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38**: 79-91.
Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S & Fujita T (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature immunology* **5**: 730-737.
Young R, Bush SJ, Lefevre L*, et al.* (2018) Species-Specific Transcriptional Regulation of Genes Involved in Nitric Oxide Production and Arginine Metabolism in Macrophages. *ImmunoHorizons* **2**: 27-37.
Young VR & Ajami AM (2001) Glutamine: the emperor or his clothes? *The Journal of nutrition* **131**: 2449S-2459S; discussion 2486S-2447S.
Yu X, Buttgereit A, Lelios I, Utz SG, Cansever D, Becher B & Greter M (2017) The Cytokine TGF-beta Promotes the Development and Homeostasis of Alveolar Macrophages. *Immunity* **47**: 903-912.e904.
Yurkovetskiy LA, Pickard JM & Chervonsky AV (2015) Microbiota and autoimmunity: exploring new avenues. *Cell host & microbe* **17**: 548-552.
Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, Granucci F & Kagan JC (2011) CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147**: 868-880.
Zhang SY, Boisson-Dupuis S, Chapgier A, Yang K, Bustamante J, Puel A, Picard C, Abel L, Jouanguy E & Casanova JL (2008) Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. *Immunological reviews* **226**: 29-40.
Zhang XO, Yin QF, Wang HB, Zhang Y, Chen T, Zheng P, Lu X, Chen LL & Yang L (2014) Species-specific alternative splicing leads to unique expression of sno-lncRNAs. *BMC genomics* **15**: 287.
Zhang Y, Zhang X-O, Chen T, Xiang J-F, Yin Q-F, Xing Y-H, Zhu S, Yang L & Chen L-L (2013) Circular Intronic Long Noncoding RNAs. *Molecular cell* **51**: 792-806.
Zhang Y, Roth TL, Gray EE, Chen H, Rodda LB, Liang Y, Ventura P, Villeda S, Crocker PR & Cyster JG (2016) Migratory and adhesive cues controlling innate-like lymphocyte surveillance of the pathogen-exposed surface of the lymph node. *eLife* **5**.
Zhao A, Yang Z, Sun R*, et al.* (2013) SerpinB2 is critical to Th2 immunity against enteric nematode infection. *Journal of immunology (Baltimore, Md : 1950)* **190**: 5779-5787.
Zhao P, Wu M, Yu H, Huang Y, Wang Y, Wang W & Yin W (2013) Serum S100A12 levels are correlated with the presence and severity of coronary artery disease in patients with type 2 diabetes mellitus. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research* **61**: 861-866.
Zhao Y, Hu X, Liu Y, Dong S, Wen Z, He W, Zhang S, Huang Q & Shi M (2017) ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway. *Molecular cancer* **16**: 79-79.
Zhou H, Chen M, Zhang G & Ye RD (2017) Suppression of Lipopolysaccharide-Induced Inflammatory Response by Fragments from Serum Amyloid A. *Journal of immunology (Baltimore, Md : 1950)* **199**: 1105-1112.